INFECTION OF CELL CULTURES WITH CAMPYLOBACTER DERIVED FROM THE PORCINE PROLIFERATIVE ENTEROPATHIES

By

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DEDICATION

To

Ifeyinwa, Chioma, Kelechukwu, Papa and Mama

with love and deep appreciation.

DECLARATION

I certify that the work presented in this thesis is my own.

Rowland Eugene Okereke.

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ABBREVIATIONS

APD	Average pore diameter
BHK	Baby hamster kidney cells
BK_{pi}	Bovine kidney cells persistently infected with Newcastle disease virus
CBA	Columbia Blood agar
CER	Chicken embryo fibroblasts into which hamster cells may have been introduced (Smith, *et al.*, 1977)
CFA	Colonisation factor
Con A	Concanavalin A
CPE	Cytopathic effects
CSM	*Campylobacter sputorum* subspecies *mucosalis*
CSM-C	CSM grown in cell free culture
CSM-T	CSM derived from adenomatous tissue
CSM-Tc	CSM derived from tissue culture cells
Ed	Editor
EDTA	Ethylenediaminetetraacetic acid
Fig	Figure
Int 407	Human intestinal epithelial cells
$LLCMK_2$	Monkey kidney cells
MDBK	Bovine kidney cells
MEM	Minimal essential medium
MSE	Measuring and Scientific Equipment
NE	Necrotic enteritis
PBS	Phosphate buffered saline
PHE	Proliferative haemorrhagic enteropathy

PIA	Porcine intestinal adenomatosis
PK	Pig kidney cells
PK_{pi}	Pig kidney cells persistently infected with Newcastle disease virus
PPK	Primary pig kidney cells
PPLO	Pleuro-pneumonia like organisms
RDE	Receptor destroying enzyme
RI	Regional ileitis
RIΩS	Rabbit anti-Ω serum
RNBGT	Rifampicin Novobiocin Brilliant Green Trimethoprim selective medium
SBA	Soya bean agglutinin
SEM	Scanning electron microscopy
STV	Saline Trypsin Versene
TIH	Transmssible ileal hyperplasia
TPB	Tryptose phosphate broth
WGA	Wheat germ agglutinin

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ABSTRACT OF THESIS (Regulation 7.9)

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Title of Thesis Infection of cell cultures with Campylobacter derived from the porcine proliferative enteropathies.

This study utilised cell cultures in an attempt to elucidate the aetiological cause of the bacterial associated porcine proliferative enteropathies. Initial work evaluated the attachment of one possible agent Campylobacter sputorum subspecies mucosalis (CSM) to cells, CSM did not adhere to PK15 cells during the first 8 hours of cellular development whereas, after 20-24 hours of growth, the majority of cells in the monolayer showed maximum bacterial attachment. Adhesion was influenced by the number of viable organisms in the inoculum, by the degree of cell to cell contact in the monolayer and by the stage of development of the cells when exposed to infection, but, in all instances, a small number of cells were refractory to bacterial attachment. Adhesion to PK15 cells was completely inhibited by rabbit anti-CSM serum, partially inhibited by wheat germ agglutinin (WGA) and soya bean agglutinin (SBA) but was not affected by any of the metallic salts tested. Attachment of CSM to in vitro preparations of pig intestinal brush borders was scanty compared with that obtained with PK15 cells and did not vary between cells from different pig genotypes.

Following attachment to PK15 cell surfaces, the bacteria were engulfed and later appeared within phagosomes or lying freely in the cytoplasm. Cytopathic effects were induced by CSM infecting doses of at least $8\log_{10}$ organisms per ml but not by $5\log_{10}$ organisms per ml. Bacterial counts of supernatant fluids and lysed cells from infected PK15 cultures, supported by immunoflourescence staining, indicated cell-associated growth of CSM but limited survival in the extracellular fluids.

Infection of tissue culture cells by CSM grown in cell-free culture (CSM-C) was compared with that of bacteria derived directly from the tissues of the proliferative enteropathies. Three types of bacteria were isolated from filtered homogenised PIA tissues. A purified inoculum of the filtrate prepared by dilution and further filtration contained organisms that stained as the intracellular organisms, grew in cell-free culture and were identified as CSM (CSM-T). Such CSM-T derived by filtration produced cytopathic changes in PK15 cells similar to those obtained with cultured CSM (CSM-C). Progressive infection of cell cultures was initiated with only $3.48\log_{10}$ per ml of CSM-T, but not with $5.26\log_{10}$ per ml of CSM-C. Irrespective of source, whether from lesions, tissue culture or cell-free cultures, it was not possible to passage CSM organisms serially in PK15 cells. Campylobacter-like forms in filtrates of PHE tissues did not multiply in PK15 cells nor produce cytopathic changes. In contrast, campylobacters and other bacteria isolated from PIA or PHE by filters of larger pore diameter multiplied in the extracellular fluids and caused rapid destruction of PK15 cells.

The novel intracellular, campylobacter-like antigen (Ω) of proliferative porcine enteropathies and hamster ileitis, which differs from the surface antigen of known campylobacters, was also detected in PK15 cells following infection with CSM-C or CSM-T organisms.

A line of PK15 cells, once-infected with CSM and subsequently self-cured, was developed but the cells showed only minor differences in characteristics from those of the parent line.

The significance and future applications of the findings of this present investigation in relation to the pathogenesis of porcine proliferative enteropathies, the diseases associated with intracellular campylobacters are discussed.

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1.1 Introduction

Many bacterial pathogens induce disease in susceptible mammalian hosts by infecting mucosal surfaces. These microorganisms must be able to survive in the mucosal or paramucosal layers to be successful pathogens, and frequently have to compete with indigenous microbial populations for access to the surfaces. They then must either be able to penetrate into the mucosa, produce toxic products that affect the epithelial cells or pass into subepithelial tissues. Attachment to the epithelial cells is a necessary prerequisite for many organisms that colonise these surfaces or penetrate into the epithelium (Smith, 1977; Gibbons, 1980; Ofek and Beachey, 1980).

The phase of attachment can be examined experimentally either in intact conventional or gnotobiotic animals, in organ culture or in non-replicating cells or cell fragments derived from the natural hosts. It is possible with certain pathogens to demonstrate attachment to some tissue culture cells, but whether such attachment involves the same mechanisms as are involved in adherence to normal host cells is not always clear. Although much is known of the attachment of certain bacteria, for example enterotoxigenic Escherichia coli, little is known about the mechanisms by which many important pathogens infect susceptible mucosae.

A number of virulent bacterial species after invasion parasitise phagocytic cells and become widely distributed in the host; the study of the pathogenic mechanisms of such organisms can be achieved by

examination of their behaviour in such cells derived from blood.

There remains a further group of diseases characterised by parasitism of the epithelium by bacteria and proliferation of host cells, a complex of such conditions affects the intestinal tract of the pig and includes those conditions described pathologically as porcine intestinal adenomatosis (PIA), necrotic enteritis (NE), regional ileitis (RI) and proliferative haemorrhagic enteropathy (PHE). These conditions are believed to possess a common basic pathological change, enterocyte proliferation. Intracellular campylobacter-like bacteria are constantly present in adenomatous or proliferating mucosal cells and the vibrioid bacterium Campylobacter sputorum subspecies mucosalis (CSM) can be frequently isolated from such abnormal tissue (Rowland et al, 1973; Lawson and Rowland, 1974; Gunnarrson et al, 1976; Lawson et al 1976 and Lomax and Glock, 1982). Despite the close association between this bacterium and abnormal cells the part played by the organism in these disease conditions remains unclear.

Attempts have been made to reproduce PIA in pigs of different immunological status by exposing them to abnormal mucosa containing intracellular vibrioid bacteria and or cultures of CSM but both these approaches have generally failed to result either in disease or the establishment and multiplication of CSM in the mucosa. Presently, there is no reliable technique for the reproduction of the disease in experimental animals, although the reported successful transmissions of PIA by Roberts et al (1977) and Lomax et al (1982a, 1982b) have implicated CSM as being important in the aetiology of this condition.

Gnotobiotic piglets, either germ-free or with a defined flora, exposed to CSM or CSM and rotavirus, resulted in both oral and intestinal colonisation by the campylobacter. However cultural, histological and ultrastructural studies failed to show the presence of significant intracellular bacterial parasitism or adenomatosis and these experiments showed that CSM alone is not readily capable in this age and type of animal of colonising pig enterocytes or inducing adenomatous change. Other agents or factors may be necessary to promote intracellular parasitism by CSM (McCartney, Lawson and Rowland, 1984).

Studies of CSM in tissue culture have shown that the bacteria attached to a variety of primary and established cell lines (Rajasekhar, 1981). Different cells responded to CSM infection in a variety of ways and CSM persisted in these cultures for varying lengths of time. The cellular abnormalities produced in these cells were associated with either the intracellular growth of CSM or the presence of intracellular killed bacteria or bacterial products (Rajasekhar, 1981). This work was a general investigation of the behaviour of CSM in tissue culture developed with a view to understanding the interaction of CSM with cultured cells and to establish satisfactory procedures for cell infection.

This present work attempts to develop certain aspects of the infection of cell cultures in an effort to elucidate the pathogenic mechanisms involved in the natural disease. In addition, in the absence of a satisfactory animal infection system it attempts to employ direct exposure of cell culture to diseased porcine tissue

rather than cultured bacteria in an effort to examine the infection process.

The literature on the use of tissue culture in the study of pathogenic mechanisms of bacterial disease has been extensively reviewed in a thesis presented in this Department (Rajasekhar, 1981) and it is not the intention of the present author to repeat that review. The survey of the literature therefore seeks to present a short synopsis of the use of tissue culture in the assessment of bacterial infections and to highlight recent studies of particular relevance to the present work.

1.2 Attachment of bacteria to surfaces of animal cells

The attachment of a bacterium to an animal cell is a specific event brought about by the molecular interaction of the bacterial adhesin and the receptor on the animal cell. Such specificity is of ecological advantage because it increases the probability that bacteria become located on a surface suitable for colonisation.

Attachment may be a random process or it may involve a directional component such as chemotaxis (Allweiss, Dostal, Carey, Edwards and Freter, 1977).

Bacteria attach to a variety of surfaces and such reactions are not confined to pathogens adhering to the cells or mammalian hosts. For instance, bacteria adhere to the surfaces of clays (Stotzky, 1974)

and glass (Marshall, Stout and Mitchell, 1971a, 1971b), the root hair surfaces of plants (Menzel, Uhlig and Weichel, 1972) the hyphae of fungi (Lockwood, 1968), the exoskeleton of copepods (Kaneko and Colwell, 1975), the gut of nematodes (Tannock and Savage, 1974), the surfaces of protozoans (Cleveland and Grimestone, 1964), surfaces of other bacterial species (Davis and Baird-Parker, 1959; Jones 1972) and a variety of primary and established cell lines (Rajasekhar, 1981; Tavendale et al, 1983).

Marine microbiologists have been aware for a long time that bacteria must stick to surfaces in order to avoid being swept away by moving streams of water (Zobell, 1943). It is only recently that it has been widely recognised that adhesion is an important ecological determinant in the colonisation of specific sites in plants and animals but is also an important early event in the pathogenesis of bacterial infections in animals and humans (Gibbons, 1977; McNeish, Turner, Fleming and Evans, 1975).

Marshall et al (1971a, 1971b) demonstrated that the attachment of bacteria to a surface consisted of two phases. The first, a reversible phase, may be equated with adsorption and is essentially an instantaneous process which depends on general rather than specific surface properties, where the binding forces are weak and the bacteria continue to exhibit Brownian movement whilst attached. The second, or irreversible phase, may be equated with adhesins and occurs when the bacterial surface contains polymers that bridge the gap between the main bacterial body and the surface (Corpe, 1970; Hirsch and Pankratz, 1970; Marshall et al, 1971a, 1971b; Fletcher and Floodgate, 1973;

Marshall and Cruickshank, 1973; Marshall, 1975).

Irreversible adhesion is characterised by the firm attachment of the bacteria and this type of interaction is typical of bacterial adhesion to animal cell receptors. It is uncertain if a distinct phase of reversible adsorption always precedes adhesion of bacteria to animal cells and indeed it seems unlikely that this occurs. The majority of bacteria that attach to animal cells have preformed adhesins and these may be the sole mechanism of adherence with bacteria migrating to the receptor site randomly. There is the possibility nevertheless that bacteria in a more natural environment produce adhesins only after they become reversibly adsorbed to the cell surface.

1.2.1 Interaction of selected bacterial adhesins and cell surface receptors

a) Vibrios

Vibrio cholerae interacts intimately with mucosal surfaces of the intestine and this association appears to be of importance in the pathogenesis of cholera (Freter, 1969). The ways and means by which V. cholerae associates with mucosae appears to involve more than one mechanism (Freter and Jones, 1976) namely, interaction with the mucus (Schrank and Verwey, 1976; Jones et al 1976) and adhesion to the brush borders of the epithelial cells (Jones et al 1976; Nelson, Clements and Finkelstein, 1976).

The adhesiveness of Vibrio parahemolyticus (Kaneko and Colwell, 1975) has been related to the production of unsheathed lateral

flagella by vibrios in response to environmental factors (de Boer, Gotten and Scheffers, 1975 ; de Boer, 1975).

b) Enterobacteriaeceae

Many different adhesive properties are exhibited by bacteria that belong to this family. In 1966 Duguid, Anderson and Campbell classified the fimbriae (or pili) produced by these bacteria, and showed that these filamentous structures are frequently responsible for adherence to surfaces.

Type 1 fimbriae and their associated adhesive activities are produced by most cultures of Escherichia coli (Duguid, 1964 and Duguid 1968), Klebsiella spp. (Thornley and Horne,1962; Duguid, 1968) and by many serotypes of Salmonella (Duguid et al, 1966).

Type 1 fimbriae confer on the bacterial cells the ability to grow as a pellicle on the surface of static broth cultures (Duguid et al 1966; Old, Corneil, Gibson, Thomson and Duguid, 1968) and to attach to a variety of animal and plant cells.

Type 2 fimbriae are produced by some salmonellae (Duguid et al 1966; Duguid 1968 and Old et al 1968) and are devoid of known adhesive or pellicle forming activities. Morphologically, they are indistinguishable from type 1 fimbriae, and are related antigenically. Old and Payne (1971) suggested that type 2 fimbriae are non-adhesive forms of type 1 fimbriae.

Type 3 fimbriae are produced by Klebsiella aerogenes and Serratia marcescens (Duguid et al 1966 and Duguid 1968). They mediate bacterial adhesion to fungal cells, plant cells, cellulose fibres and glass but not to animal cells unless these are modified by chemical or physical means.

Type 4 fimbriae of Proteus spp (Hoeniger, 1965 and Silverblatt, 1974) cause mannose-resistant haemagglutination of selected erythrocytes (Sheddon, 1962; Duguid, 1968). The adhesive properties of type 4 fimbriae produced by Pr. mirabilis may contribute to the organism's ability to cause pyelonephritis (Silverblatt, 1974).

Colonisation of the mucosal surface of the small intestine by enterotoxigenic E. coli occurs without tissue invasion and is a necessary early stage in the pathogenesis of diarrhoea (Moon, 1978; Boedeker, 1982; Gaastra and de Graaf, 1982). This colonisation appears to be dependent upon the ability of the involved strains to adhere to the villus epithelium. Adhesion to epithelial surfaces is mediated by specific heat-labile surface antigens which can be seen in electron micrographs as fimbriate fine filamentous structures (Stirm, et al, 1967 a,b ; Burrows et al, 1976; Isaacson, 1977), attachment being achieved by the interaction of the tips of many of these filamentous structures with specific mucosal receptors (Knutton et al, 1984). These bacterial structures are distinct from the classical Duguid types of fimbriae and are named K88 and 987P in porcine-specific E. coli (Ørskov et al, 1964; Stirm et al, 1967 a,b; Smith and Linggood, 1971; Jones and Rutter, 1972; Nagy et al, 1977; and Isaacson et al, 1978), the K99 pilus in bovine-, ovine- and some

porcine-specific E. coli. (Smith and Linggood, 1972; Ørskov et al, 1975; Burrows et al, 1976; Moon et al, 1976; Myers and Guinee, 1976), and the colonisation factors (CFAI and CFAII) of E. coli pathogenic for humans (Evans et al, 1975; Ryder et al, 1976; Evans and Evans 1978; and Evans et al, 1978).

Field and laboratory observations indicate that these colonisation-promoting antigens account in part for pathogenicity and host specificity in E. coli. (Smith and Linggood,1971 and Ørskov et al, 1975). These antigens share some similar properties. For example, they are all plasmid associated (Evans et al, 1975; Smith and Linggood, 1972), and each is detectable as a mannose-resistant haemagglutinin (Stirm et al, 1967 a, b; Tixier and Gouet, 1975; Burrows et al, 1976; Evans et al, 1977; Ørskov and Ørskov, 1977).

The K88 antigen is a protein that takes the form of numerous fine filaments covering the surface of the bacterial cell (Stirm et al, 1967a, 1967b). This antigen is an essential virulence factor of certain enterotoxigenic E. coli in conventionally reared piglets (Jones and Rutter, 1972). The K88 gene is carried by a transmissible plasmid which may be spontaneously lost upon subculture of K88-positive cultures in the laboratory (Ørskov and Ørskov, 1966; Smith and Linggood, 1971). The association of this antigen with specific adhesion seems to be limited to E. coli serotypes because transfer of the K88 plasmid to salmonellae does not produce an adhesive effect. Some piglets do not inherit specific receptors for the K88 antigen and are resistant to infection by such strains. Porcine $K88^-$ E. coli strains and non-enterotoxigenic E. coli have not been shown to

adhere to intestinal epithelial cells *in vitro* although *in vivo* studies indicate the presence of as yet undescribed adhesins. K88-positive *E. coli* adhere to the brush borders prepared from small intestinal epithelial cells from most but not all pigs (Sellwood *et al*, 1975; and Snodgrass *et al*, 1981). The two phenotypes are the product of two alleles of a simple locus which are inherited in a single Mendelian manner with adherence (S) being dominant over non-adherence (s) (Gibbons *et al*, 1977; Sellwood and Kearns, 1979). Susceptibility to diarrhoea caused by both natural and experimental infections with K88-positive *E. coli* is limited to piglets of adherent phenotypes (Rutter *et al*, 1975 and Sellwood, 1979).

The K99 antigen is a protein which is encoded on a 58-megadalton plasmid in the type strain B41 and is composed of a simple repeating protein subunit of 18.2 kilodaltons (Isaacson *et al*, 1981). The expression of this antigen is subject both to genetic regulation and catabolite repression (Isaacson, 1980). For example, expression is repressed by glucose and this can be reversed by the addition of cyclic-AMP to the culture (Isaacson, 1980). The amino acid L-alanine (de Graaf *et al*, 1980) and growth at 18°C (de Graaf *et al*, 1980; Isaacson, 1980) also repress K99 expression, but aeration promotes expression (Isaacson, 1980).

Studies by Evans and Evans , (1978) show that CFAI and CFAII mediate mannose resistant haemagglutination of human group A and bovine erythrocytes respectively. These colonisation factors were never found on the same serotype of *E. coli* but 98% of *E. coli* belonging to the frequently isolated serogroups 06, 08, 015, 025, 063 and 078 demonstrated

either CFAI or CFAII (Evans and Evans, 1978). E. coli belonging to serogroups 06 and 08 do not possess CFAI but possess CFAII and preliminary evidence indicates that this antigen is a distinct form of fimbriae (Evans and Evans, 1978). CFAII-positive E. coli, such as strain PB-176, possess fimbriae when grown on CFA agar and haemagglutinate bovine erythrocytes, whereas CFAII-negative derivatives such as PB-176-P neither produce fimbriae on CFA agar nor haemagglutinate bovine erythrocytes.

Recently, a new human colonisation factor (CFAIII) was reported which did not show mannose-resistant haemagglutination with either human or bovine erythrocytes (Honda et al, 1984). There were antigenic and molecular weight differences between this colonisation factor and CFAI and CFAII. The colonisation ability of CFAIII was confirmed by animal experiments in suckling mice and infant rabbits.

c) Streptococci

Fibrillar layers on the surface of streptococci have been demonstrated (Gibbons et al, 1972; Lai et al, 1973; Nalbandian et al, 1974) and the close association between this fibrillar material and human oral epithelial cell surface suggests that adhesion is mediated by this surface layer (Gibbons et al, 1972; Liljemark and Gibbons, 1972). Removal of the fibrillar material results in the inability of streptococci to attach to epithelial cells (Ofek et al, 1975) and loss of the ability to colonise the rodent oral cavity (Ellen and Gibbons, 1972).

Streptococcus pyogenes produces surface fibrillar material (Ellen and Gibbons, 1972) that is associated with the selective adherence properties of this streptococcal cell (Ellen and Gibbons, 1974; Beachey and Ofek, 1976; Ofek et al, 1975; Gibbons, 1977). S. agalactiae adheres to the epithelial cells of bovine udder (Frost, 1975) as well as human embryonic amnion cells in culture (Goldschmidt and Panos, 1984).

d) Mycoplasma

Species of mycoplasma can adhere to tissue culture cells, leukocytes and the surfaces of tracheas in organ culture (Collier and Clyde, 1971; Stanbridge, 1971; Collier and Baseman, 1973 and Muse et al, 1976). Mycoplasmas also exhibit haemagglutinating activity (Manchee and Taylor-Robinson, 1968) and the ability to attach to and subsequently grow on glass surfaces (Bredt, 1968).

Some mycoplasmas have distinct organelles located at one pole of the cell (Collier and Clyde, 1971). Electron microscopic studies demonstrated that when mycoplasmas attach to tissue cell surfaces, the organelle is located adjacent to the tissue cell (Muse et al, 1976) and probably acts as the adhesins. However, the adhesins of mycoplasmas are not identical and there is evidence that the polar organelles occur in different forms, and there are also intra- and inter-species differences in their haemagglutinating and other adhesin activities (Manchee and Taylor-Robinson, 1968; Sobeslavesky et al 1968). All of these features indicate the diversity of these important agents. Adhesive properties probably facilitate colonisation of the surfaces of the animal body. The few pathogenic types of mycoplasma that have been examined are virulent and invariably adhesive (Clyde,1975).

e) Neisseriaceae

There are four colony types of Neisseria gonorrhoeae designated 1, 2, 3 and 4. Types 1 and 2 produce lateral fimbriae (Swanson, 1972) whilst types 3 and 4 do not produce fimbriae (Jephcott, Reyn and Birch-Anderson, 1971; Swanson, Kraus and Gotschlich, 1971). Subsequent studies with a variety of animal cells established that fimbriate gonococci are significantly more adhesive than non-fimbriate gonococci (Punsalang and Sawyer, 1973; James-Holmquest, Swanson, Buchanan, Wende and Williams, 1974; Ward, Watt and Robertson, 1974; Swanson, Sparks, Young and King, 1975) and that fimbriate gonococci adhere to the mucosal surface of fallopian tubes (Ward et al, 1974) and cause haemagglutination (Punsalang and Sawyer, 1973; Chan and Wiseman, 1975).

Neisseria meningitidis produce fimbriae similar in dimensions to the fimbriae of N. gonorrhoeae (Devoe and Gilchrist, 1974).

Fimbrial and adhesive activities however are not confined to pathogenic Neisseriaceae, and species of Neisseria considered to be commensals of mucosal surfaces produce adhesive surface appendages that exhibit both intra- and interspecies differences in form and adhesive properties (Wistreich and Baker, 1971).

f) Lactobacilli

The lactobacilli of the crop of the domestic chicken are adhesive and attach to crop epithelial cells in vitro (Fuller, 1973) and in vivo (Fuller and Turvey, 1971). Only lactobacilli isolated from birds were found to adhere to crop epithelial cells whereas lactobacilli of mammalian origin did not do so (Fuller, 1973).

Adhesive lactobacilli appear to attach to the epithelial surface by means of fibrillar material (Takeuchi and Savage, 1973; Fuller and Brooker, 1974; Brooker and Fuller, 1975).

g) Corynebacteria

Many corynebacteria produce surface appendages that vary in size and morphology depending upon the species (Yanagawa and Honda, 1976).

The adhesive properties of Corynebacterium renale and C. diphtheriae are associated with such structures (Honda and Yanagawa, 1974; Yanagawa and Honda, 1976). C. renale adheres to tissue culture cells (Honda and Yanagawa, 1975) and to the bladder wall of the mouse and it is very possible that C. renale colonises the urinary tract of cattle and causes disease in much the same way because of these adhesins.

h) Campylobacters

Campylobacter jejuni attaches to HeLa 229 and Int 407 cells and this takes place after impact by centrifugation but little or no attachment occurs without centrifugation (Newell and Pearson, 1981).

Subsequent studies (McBride and Newell, 1983) with Int 407 cells grown in monolayers using aflagellate (FLA^- MOT^-), and non-motile (FLA^+ MOT^-) variants selected from parent C. jejuni (FLA^+ MOT^+) indicate that the aflagellate variant attaches relatively poorly whilst the non-motile flagellated variant attaches best, with the wild-type less successful. It is suggested that the flagella has an adhesin which gives flagellated organisms an adhesive advantage over

aflagellated variants (McBride and Newell, 1983).

In contrast, aflagellate variants attached significantly better than the wild-type or non-motile variants to buccal or red cells in suspension (McBride and Newell, 1983). There seems therefore to be a second adhesin(s) on the surface of C. jejuni and the interaction between this adhesin (s) and the target cell is hindered by flagella.

Experimental 5-day-old infant mice orally infected with these three strains were colonised following attachment of all 3 strains to the microvilli of intestinal epithelial cells. The wild-type was isolated from the small intestine or faeces for up to 30 days after infection, whereas only a few aflagellate variants were recovered after 7 days. Nevertheless, the non-motile variant colonised the gut as efficiently as the wild-type (Newell et al, 1983). C. jejuni has also been shown to adhere to pig brush borders but the ratio of bacteria to brush borders had to exceed 100:1 for any apparent adhesion to take place. The bacteria adhered to both the brush borders and the adjacent cell fragments (Naess et al, 1983).

Studies of the relationships of CSM to mammalian and avian-derived cells in both primary and continuous cultures (Rajasekhar, 1981) showed that CSM attached specifically with varying intensity to certain cells. Whilst cells derived from many species had the capacity to bind CSM, certain species of cell cultures proved refractory. CSM attached more vigorously to primary pig kidney (PPK), pig kidney (PK) and pig kidney persistently infected with Newcastle Disease Virus (PK_{pi}) than to the other cells tested. Bacteria

adhered less densely to bovine kidney (MDBK), bovine kidney cells persistently infected (BKpi), baby hamster kidney (BHK) and HeLa cells than to pig-derived cells. Vero cells, $LLCMK_2$ and chicken embryo fibroblasts were refractory on primary exposure to the attachment of CSM.

Viable and air-killed CSM have been shown to attach differently to PK15 cells. Viable bacteria attached densely to the periphery of the cells while fewer air-killed bacteria attached and were more widely dispersed over the surface and showed no particular preference for periphery of the cell (Rajasekhar, 1981).

From these experiments motility appears to play an important role in the attachment of Campylobacter to cells in preformed monolayers and although in C. jejuni the flagella carry an adhesin with a specificity for a receptor expressed on the cell there does not appear to be any direct evidence for this in the case of CSM (Newell et al, 1983; Rajasekhar, 1981).

In vitro studies on CSM and Campylobacter have only been carried out by a few workers during the last decade and for this reason the literature is scant.

1.3 Host and tissue specificity of bacteria

Many species of bacteria show a degree of specificity for the hosts and tissues they infect, and the attribute which confers this function is generally important in the pathogenesis of disease.

The specific mechanisms of infection depend therefore not only on the presence of a specific character of the microbe but also on the fact that the host itself provides the necessary conditions for the mechanisms to be effective if the host is to be susceptible. Host susceptibility may also be moderated by the hosts immune status or other mechanisms of resistance.

The idea of the specificity of the interaction between bacteria and host is suggested by the species specificity of certain bacterial infections. For example, gonococcal infections are limited to humans (Johnson, Taylor-Robinson and McGee, 1977) and diarrhiagenic $K88^+$ *E. coli* only infects pigs (Jones and Rutter, 1974).

Bacterial surface components play a prominent role in mediating selective adherence to the mucous surface at the initiation of infection (Gibbons, Spinell and Skobe, 1976; Gibbons and van Houte, 1975). The colonisation of some tissues in preference to others by pathogenic bacteria modulates the disease-causing abilities of the organism in the host. For example, *Streptococcus pyogenes* attaches to human pharyngeal cells better than *E. coli*. This correlates with the fact that *S. pyogenes* and not *E. coli* commonly colonises the oral cavity (Ellen and Gibbons, 1974). *Vibrio cholerae* and some pathogenic *E. coli* are able to colonise the epithelium of the upper bowel, whereas *Shigella flexneri* adheres to colonic cells rather than those of the

upper bowel (Smith and Pearce, 1972). In all these instances of infections with S. pyogenes, V. cholerae, E. coli or S. flexneri important lesions may be expected at the site related to colonisation.

In in vitro experiments, the varied intensity of attachment of CSM to cells derived from various species also appears to indicate a degree of host specificity. Thus, CSM attaches better to pig kidney cells than to bovine or avian-derived cells (Rajasekhar, 1981). No information is directly available on the attachment of CSM to other types of pig cells which are likely to be relevant in natural infection as the only other porcine cell cultures examined by Rajasekhar (1981) were red blood cells.

1.4 Events that take place after irreversible adhesion of bacteria to host cells

1.4.1 Entry of bacteria into host cells

An essential feature in the pathogenesis of diseases due to intracellular parasites is the ability of the pathogen to enter and replicate within the appropriate host cell after initial attachment.

Particles normally enter cells by phagocytosis so that the particle is enclosed in a phagocytic vacuole but there are other methods of cell entry. Ultrastructural studies indicate that some bacteria adsorb to the cell surface and enter the cytoplasm directly after inducing local damage of the plasma membrane. Shigella and pathogenic Salmonella appear to enter intestinal epithelial cells

in this way, and other bacteria show the same behaviour in tissue culture cells. Protozoa can utilise their own lysosomal enzymes to penetrate host cells. Trypanosomes, Toxoplasma gondii and Entamoeba histolytica may enter susceptible cells by active penetration, and the relevant pole of the parasite has vesicles containing lysosomal enzymes.

Experiments in rabbits have shown that in the early stage of intestinal infection with virulent Yersinia pseudotuberculosis, organisms can be detected in mucosal epithelial cells and it is suggested that bacteria may penetrate epithelial cells to reach the lamina propria and lymph follicles (Une, 1977). Y. pseudotuberculosis also has the capacity to penetrate epithelial cells in vitro and HeLa cells rapidly ingest the bacteria by a phagocytosis-like procedure that has phases of attachment and engulfment (Bovallius and Nilsson, 1975; Brunius, 1980).

Electron microscopic studies of the penetration of salmonellae and E. coli into intestinal epithelia (Takeuchi, 1967; Takeuchi et al, 1968 and Staley et al, 1969) showed that these organisms pass into epithelial cells by a process very similar to phagocytosis (Staley et al, 1969). As the bacteria approach the apical surface of the intestinal epithelial cells the brush borders begin to degenerate in the immediate region of the bacteria. As the bacteria pass through the degenerating brush border and into the cells they are surrounded by inverted cytoplasmic membranes. Bacteria within the cell cytoplasm are enclosed in membrane-bound vacuoles similar to phagocytic vacuoles seen in macrophages and other phagocytic cells (Takeuchi, 1967;

Staley et al, 1969). The salmonellae and some strains of E. coli (Staley et al, 1970) surrounded by the vacuole membranes pass intact through the epithelial cells and into the lamina propria.

Studies with rickettsiae have shown that in some species the dynamics of entry into cells is not by way of phagocytosis but depends upon the properties of viable and metabolising organisms. This penetration is optimal under conditions which enhance oxidative phosphorylation by rickettsial suspensions but is depressed if the rickettsial metabolism is impeded by dinitrophenol, cyanide or azide.

The method of entry of CSM into cells in culture is not completely clear but it is thought that the organism becomes partially trapped, encircled and engulfed by the pseudopodia or cellular surface processes (Rajasekhar, 1981). Host cell membrane changes are apparent and the uptake of bacteria results both in bacteria lying freely within the cytoplasm and trapped within phagosomes.

1.4.2 Intracellular multiplication of bacteria

The pathogenesis of invasive bacteria such as Shigella begins with penetration of intestinal epithelial cells (La Brec, Schneider, Magnani and Formal, 1964) followed by intracellular multiplication (Formal, La Brec, Kent and Falkow, 1965).

In cell culture, intracellular multiplication of bacteria has been used to evaluate the pathogenic potential of various bacteria including Shigella flexneri (Calabi, 1970; Gerber and Watkins, 1961; Hale and Bonventre, 1979), E. coli (Mandell, 1973);

Salmonella typhimurium (Kihlström, 1977; Kihlström and Edebo, 1976); Legionella pneumophilia (Wong et al, 1980; Horwitz and Silverstein, 1980, 1981; Daisy et al, 1981); Yersinia enterocolitica (Devenish and Schiemann, 1981) and Campylobacter sputorum subspecies mucosalis (Rajasekhar, 1981).

Studies on the interaction of Yersinia enterocolitica with cells in culture have shown that common human serotypes of this organism multiply intracellularly (Lee, McGrath, Carter and Eide, 1977; Maki, Gronroos and Vesikari, 1978; Maruyama, Une and Zen-Yoji, 1979). Legionella pneumophilia, the aetiological agent of Legionnaires' disease has been shown to multiply intracellularly in normal human embryonic lung fibroblasts (Wong et al, 1980).

The growth of Rickettsia rickettsii in a mouse tumour cell line (Bozeman, Hopps, Danauskas, Jackson and Smadel, 1956), revealed that the respiration and growth of the mouse cells was not modified by infection and that the multiplication of the rickettsiae continued even when the growth of the host cells had been inhibited by colchicine.

Some cells in culture are able to support intracellular growth of CSM (Rajasekhar, 1981) and an unusual feature of this infection was the relatively long period over which infected cells remained viable.

1.4.3 Killing or survival of bacteria in phagocytic vacuoles

The phagocyte is an important part of the host defences that can operate without delay against invading microorganisms after the

epithelial surface has been breached.

As a result of phagocytosis, microorganisms are enclosed in membrane-lined vacuoles in the cytoplasm of the phagocytic cell and subsequent events depend on the activity of the lysosomes. These move towards the phagocytic vacuoles, fuse with its wall to form a phagolysosome and discharge their contents into the vacuole thereby lowering the pH of the vacuole to acid (pH 3.5 - 4.0). This acidity has some antimicrobial effect and initiates the intracellular killing and digestion of the microorganism. Bacteria are killed and digestion proceeds, first of the bacterial cell wall components and subsequently of the contents of the bacterial cell. In some cases after phagocytosis microorganisms resist killing and digestion. These microorganisms are thought either to be resistant to the bactericidal conditions which develop in this microenvironment or they prevent delivery of bactericidal products to the vacuole. These microorganisms are specialist intracellular parasites and some of them depend upon their persistence in phagocytes, as in infections with Mycobacterium leprae or Mycobacterium tuberculosis, for their pathogenic effect.

The encounter between the microorganism and the phagocytic cell is a central feature of infection and pathogenicity. Phagocytes are designed to ingest, kill and digest invaders and the course of the infection depends on the success with which this is carried out. Virulent microorganisms seem to have developed a variety of devices for countering or avoiding the antimicrobial action of phagocytes.

In PIA the intracellular campylobacters usually lie free

within the cytoplasm of the epithelial cells situated in the apical cytoplasm. During this period, therefore, the organisms are not affected by the phagocytic activity of the epithelial cell, which they appear to have eluded, nor are they presented to the "professional" phagocytic cells of the lamina propria. Organisms within phagosomes have been described but opinions differ as to whether these represent healing and resolution of the lesion (Roberts, 1978) or whether they occur at the time of initial uptake of the bacteria (Kurtz, Soto and McAllister, 1980).

Information on other Campylobacter infections is not helpful nor informative as few of the other infections described have a pronounced phase of epithelial cell parasitism. Campylobacter fetus has a glycoprotein antiphagocytic surface component designated antigen-a which renders bacterial cells refractory to phagocytosis except in the presence of specific serum (Winter and McCoy, 1978), but the relevance of such a component, if it exists, for the campylobacters of adenomatosis is clearly highly questionable.

1.4.4 The sequelae of host infection by bacteria

Bacterial pathogens possess unique properties by which they can overcome the host defences in order to multiply and, consequently, damage the host. Injury is often the consequence of the metabolic activity of virulent microbes multiplying within the host tissues. Bacteria utilise the body tissues and fluids as sources of energy and as nutrients for the synthesis of microbial cellular constituents. This process often involves microbial breakdown of host constituents resulting in tissue damage. In addition some pathogens release

enzymic by-products of their metabolism, some of which are highly toxic to the host. These exotoxins specifically and often irreversibly damage vital functions of the host cells.

If the damage caused by infecting bacteria is controlled and the microorganisms eliminated from the tissue, then the process of cell recovery follows. But adenomatosis appears to be somewhat unique in that, to some extent, the affected tissues show little cell damage or death. The lesion is predominantly made up of immature proliferating cells which do not appear to be damaged and are otherwise typical of immature epithelial cells except for the presence of intracellular bacteria. In the case of PHE and NE there is necrosis or damage but, as these changes are probably superimposed on the initial lesion, it is possible that they involve a separate mechanism.

Cell culture studies (Rajasekhar, 1981) appear to indicate that CSM is capable of attachment to and ingestion by cells. It also persists intracellularly for prolonged periods and this brings about profound alteration of cell function but without immediate death of these cells and without cell proliferation. These features are to some extent similar to cell parasitism in adenomatosis where cells parasitised by campylobacters appear relatively unharmed although the two types of infection differ in their proliferative response.

1.4.5 Problem of extracellular growth of bacteria in cell cultures

The multiplication of bacteria in the extracellular fluid is one of the problems encountered with bacterial infection of tissue culture. This extracellular growth tends to persist and is often

associated with degenerate changes in the infected cell culture monolayers. Intracellular organisms therefore may be derived from phagocytosis of extracellular bacteria and clearly ultrastructural examination may not be capable of differentiating these two situations with clarity. In the same way attempts to assess intracellular growth by cultural recovery of the bacteria are often likely to be confused by contamination from extracellular bacteria. Attempts to resolve these difficulties by use of antibiotics to control extracellular bacterial growth are not without their critics. These problems have been fully discussed by Rajasekhar (1981) and are inherent difficulties in the assessment of many non-obligate intracellular bacteria.

CSM do not grow in cell culture fluid with or without non-permissive cells but grow in tissue culture fluid in hydrogen microaerophilic atmosphere, or in tissue culture fluid with permissive cells (Rajasekhar, 1981). This tends to indicate with electron micrograph and immunofluorescent staining that the cell lines examined showed CSM persistence related to intracellular multiplication.

1.5 The proliferative enteropathies and their association with campylobacters

1.5.1 Porcine Intestinal Adenomatosis (PIA)

Porcine intestinal adenomatosis (PIA) is a transient proliferation of the intestinal mucosa of the weaned pig (Biester, Schwarte and Eveleth, 1939; Dodd, 1968; Rowland and Rowntree, 1972) in which there

is constant presence of an intracellular bacterium (Rowland, Lawson and Maxwell, 1973; Rowland and Lawson, 1974). Subsequent studies by Lawson et al (1975b) showed that a morphologically similar campylobacter, characterised as C. sputorum subspecies mucosalis (CSM) can be recovered from the lesions in large numbers, up to 10^8 per gm in the diseases mucosa. The bacteria are largely confined in the intestine to the abnormal tissue, being absent or present in restricted numbers in the non-adenomatous intestine of affected animals. Smaller numbers of organisms may be recovered from the chyme, while recovery from faeces using the available selective media is irregular. The organisms may be recovered from the oral cavity of pigs and this colonisation has been shown to persist for a period of some 8 weeks in both experimental and natural infections (Lawson et al, 1975a; Roberts et al, 1980a).

Although the successful transmissions of PIA reported by Roberts et al (1977) and Lomax et al (1982a, 1982b) have strongly implicated CSM as being important aetiologically, the intracellular parasitism by CSM seems to require the help of other agents or factors, because CSM alone is not readily capable of infecting pig enterocytes or inducing adenomatous change (McCartney et al, 1984).

A new species of Campylobacter, Campylobacter hyointestinalis (CHI) was isolated from 18 of 27 and 27 of 29 cases of swine proliferative ileitis by Gebhart et al (1983) and Chang et al (1984) respectively. CSM was also isolated from many of these cases and, in the former study, 11 of the cases yielded both organisms.

Although described as a new species by Gebhart et al (1983), similar bacteria were described by Lawson and Rowland (1974) as

Campylobacter coli, and both Gebhart (personal communication, 1984) and Terzolo (personal communication, 1984) have found these organisms to be very closely related. The strain isolated by Lawson and Rowland (1974) has been designated as NCTC 11562 but whether this species designation of CHI for this group of bacteria will be retained is not clear. These bacteria are more widely distributed than CSM and are commonly isolated from calves (Terzolo, 1984 personal communication). The same author has examined the pathogenicity of CHI in gnotobiotic lambs, a species which also suffer from a proliferative enteropathy, and found that they had little effect. Studies by Almashat and Taylor (1981) on an organism incorrectly designated Campylobacter faecalis and which was, in reality, CHI (Terzolo, 1984 personal communication) suggests that it may have some minor pathogenicity for calves. Rajasekhar (1981) employed strain NCTC 11562 (124/73-A4) in his studies of infection of various cell culture systems.

1.5.2 Other related conditions

Necrotic Enteritis (NE), in which the altered mucosa undergoes a coagulative necrosis and regional ileitis (RI), in which much of the mucosa is replaced by granulation tissue, are other conditions of pigs from which CSM have been isolated. Both show intracellular vibrios in the undamaged surrounding epithelium and CSM may be recovered along with comparable numbers of catalase-positive vibrios (Rowland and Lawson, 1975).

Another clinical condition called proliferative haemorrhagic enteropathy (PHE) is characterised by thickening of a varying

length of terminal ileum as well as oedema of the associated mesentery, and mainly affects young adult animals of over 4 months of age. The proliferative intestinal lesion is complicated by substantial haemorrhage which gives the disease its name (Rowland and Rowntree, 1972). Isolation of CSM from these lesions is difficult and the organisms are few in number compared with those that may be isolated from PIA (Love and Love, 1979; Lawson et al, 1979). It is possible that this clinical condition may involve bacteria other than CSM, although Roberts (1978) showed that the intracellular organisms in PHE are often degenerate and such damage may account for the difficulty experienced in isolating the bacteria.

Immunofluorescence studies of parasitised cells show particulate fluorescence within the mucosal epithelium and also within lumen debris (Rowland and Lawson, 1975).

1.6 The aetiology of the proliferative enteropathies

In pigs with PIA the greatest concentration of CSM occurs in association with adenomatous tissues, and the areas of normal intestinal mucosa do not harbour the organism. There is no direct evidence that the intracellularly dividing campylobacters seen by electron microscopy, bring about the adenomatous change. However, the possibility exists either that, the epithelial cells are preferentially parasitised by the bacteria and that a change is subsequently induced in the gland cells, or that adenomatous epithelium developing as a result of some other influence specifically phagocytoses this organism from a postulated terminal population (Lawson et al, 1975a). Rowland and Lawson (1974)

revealed by electron microscopy, the presence of dividing campylobacters within the host cell cytoplasm and speculated that immature epithelial cells can become infected by CSM which persists intracellularly and thereby permits the infection to spread within the epithelium. The bacteria are not bound by a membrane but lie free in the apical host cell cytoplasm (Rowland and Lawson, 1974).

Lawson et al (1981) suggested that the energy required for the multiplication of intracellular CSM is derived from the host and that hydrogen or other electron donors may play a role in the intracellular growth of the parasite.

Necrotic enteritis was thought at one time to be a common manifestation of salmonellosis or to be caused by panthothenic acid or nicotinic acid deficiency (Chick et al, 1938; Davis and Freeman, 1940). It is uncertain whether these manifestations represent a different disease with a similar pathological presentation or whether the underlying disease described in previous years is the same condition with which we are familiar today.

The residual epithelium shows the essential features of PIA and therefore the underlying pathogenesis of the proliferative lesion is not likely to be significantly different from that of PIA.

Proliferative lesions of the small intestine have been reported in several species (Cross et al, 1973 and Seronde, 1970) and those of comparative interest include transmissible ileal hyperplasia (TIH) or proliferative ileitis of hamsters (Frisk and Wagner, 1977). This is an enzootic disease which resembles PIA histologically. The aetiology of TIH remains unknown but filtration studies indicates

that the causative agent (s) is comparable with a bacterium (Jacoby et al,1975) and Gram negative curved bodies have been detected in the apical cytoplasm of hyperplastic cells. There is also ultrastructural evidence that bacteria colonise the ileal epithelium in both naturally occuring and experimental TIH (Johnson and Jacoby, 1978 and Wagner et al, 1973).

Murine colonic hyperplasia is another proliferative condition that may be induced by infection with certain strains of Citrobacter freundii, (Barthold, et al 1976) but infection here is of the mucosal surface and intracellular parasitism is not a feature.

Regional ileitis of lambs has also been described (Cross et al, 1973 and Wensvoort, 1962) and organisms with vibrio morphology have been found in the epithelial cells (Hoorens et al, 1977). In a subsequent study of an animal with diphtheresis of the intestinal mucosa, Vandenberghe and Hoorens (1980) isolated two strains of catalase-negative campylobacter resembling CSM and C. jejuni respectively.

1.7 Clinical and pathological signs of the proliferative enteropathies

PIA is a proliferative enteropathy of weaned pigs which resembles a transient neoplasm. The lesions consist of immature proliferating epithelial cells (Rowland and Lawson, 1975). Weaned pigs suffering from PIA may show little appetite and insignificant weight gain. In some cases pigs can be in extremely poor condition and at the point of emaciation. Recovery can be rapid however, and such animals demonstrate

no lesions at slaughter (Rowland and Rowntree, 1972; Roberts et al, 1977). Although PIA was first reported about 50 years ago (Biester and Schwarte, 1931) only recently have there been serious attempts to implicate a bacterial agent, and Rowland et al (1973) were the first to describe CSM which appeared to be intimately associated with the adenomatous tissue.

Rowland and Lawson (1975) observed that the distribution of the damaged tissue in NE closely corresponds to that of PIA. The ileum shows marked thickening and coagulative necrosis of much of the mucosa. In many cases massively enlarged necrotic glands can be seen. The surrounding intact mucosa is markedly hyperplastic. Diarrhoea is a likely symptom of NE with the faeces often containing flecks of necrotic mucous membrane. Pigs affected with NE may become depressed or die rapidly.

Regional ileitis is a functional obstruction of the lower ileum associated with proliferation of the granulation tissue in the lamina propria and submucosa, and a massive muscular hypertrophy of the wall of the affected areas of the bowel. Histologically, the lumen surface is often covered by a layer of necrotic debris (Rowland and Lawson, 1975).

Proliferative haemorrhagic enteropathy (PHE) is more commonly encountered in adult animals although cases can occur in pigs of all ages over three weeks (Rowland and Rowntree, 1972). Pigs with this condition pass large quantities of black, foul-smelling, altered blood in the faeces. In many cases death is sudden and affected

animals may be found dead without showing clinical symptoms. PHE is characterised by thickening of a varying length of terminal ileum and oedema of the associated mesentery. There is marked epithelial hyperplasia in which immaturity of the cells is a feature and, as a result, only a few goblet cells are present.

1.8 Purpose of the present investigation

Ever since the demonstration of intracellular CSM in adenomatous epithelium (Rowland et al,1973) considerable doubt has remained about the part played by this organism in the production of these abnormal intestinal cells.

A number of attempts have since then been made to reproduce the disease in experimental animals with a view to understanding the pathogenesis of the condition. These can be summarised as follows:-

i) Exposure of conventional pigs to diseased mucosa has rarely resulted in disease but use of high health-status pigs appears to be more encouraging (Lomax et al, 1982). However this work requires to be repeated since not all workers have access to such class and age of animal, and the microbiological observations are scanty.

ii) Infection of conventional pigs with CSM has not resulted in significant colonisation or adenomatous change.

iii) Infection of gnotobiotic piglets with CSM or CHI has resulted in prolonged colonisation but without significant parasitism of the

cells and little evidence of visible adherence to enterocytes.

As a consequence of these unsuccessful attempts to reproduce PIA alternative ways to investigate the disease process were sought and the primary aims of this present work have been chosen as follows:-

a) To confirm the procedures developed by Rajasekhar (1981) for infecting cell culture systems with cultured CSM.

b) To investigate the phenomenon of attachment of CSM to cell cultures with a view to assessing its significance in the natural diseases, and in a search for features which might modify attachment.

c) To expose cell culture systems to intracellular campylobacters derived directly from pathological specimens, and to compare the mechanisms of attachment, penetration and multiplication with those of cultured CSM.

It was also hoped that such experiments might elucidate some specific areas of doubt, namely:-

i) Whether diseased mucosa is more successful than bacterial cultures in initiating the disease.

ii) To search for the presence of additional campylobacters such as CHI or other as yet undescribed species in the lesions of PIA that might also infect cultured cells.

iii) To compare the results obtained from infection of cell cultures with material derived from PIA and PHE.

iv) To examine infected cultured cells for the presence of agents other than campylobacters.

CHAPTER 2.

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General Materials and Methods

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2.1 Bacteriological media

All media where appropriate were prepared according to the relevant manufacturers instructions.

i) Columbia blood agar (CBA):

7% defibrinated horse blood in Columbia agar base (Oxoid CM 331).

ii) CBA slopes:

10ml or 30ml of CBA in 25ml McCartney bottles and 100ml medical flats respectively.

iii) Tryptose phosphate broth (TPB):

Oxoid CM 283.

iv) Diphasic media consisted of the appropriate CBA slope with an overlay of 5ml or 20ml TPB.

v) Campylobacter selective medium (RNBGT) consisted of a nutrient base (Oxoid CM271) with 5% (v/v) horse blood, Novobiocin (Sigma* - N1628) at 5μg/ml, Trimethoprim (Sigma - T7883) at 5μg/ml, Rifampicin (Sigma - R3501) at 5μg/ml and brilliant green (G.T. Gurr Ltd., London) at 1/60000.

*Sigma Chemical Company Ltd., Dorset, England.

vi) Mycoplasma (PPLO Agar):

Bacto PPLO Agar (Difco, 0412-01).

2.2 Cell culture media

Dulbecco's modification of Eagle's medium (Dulbecco and Freeman, 1959), Eagle's minimum essential medium (MEM) with Hanks' salts (Eagle, 1959) and medium 199 with Earle's salt (Morgan, Morton and Parker, 1950) were obtained from Gibco-Biocult, Paisley, Scotland.

All were supplemented with sodium bicarbonate (4.4% w/v solution) and L-glutamine (200mM) at final concentrations of 0.16% and 1% respectively.

Also used was Eagle's MEM with Earle's salts modified for suspension cultures (S-MEM) supplemented with L-glutamine and 0.3% methyl cellulose. Pooled calf or foetal calf serum sterilised by positive filtration through Millipore membrane filters of 0.22μm A.P.D. and inactivated at 56°C for 30 minutes, was added to all media at 1% for maintenance and 10% for growth unless stated otherwise.

2.3 Reagents

i) Phosphate Buffered Saline (PBS)

Dulbecco's 'A' phosphate buffered saline (Oxoid, BR 14a) 0.01M pH 7.3

ii) Trypsin

A stock solution of 1% trypsin (Difco, 0152-15) was prepared by dissolving trypsin overnight at 4°C in PBS. The solution was sterilised by filtration through sintered glass and stored at -30°C in 200ml amounts. Working dilutions were prepared from the stock solution in PBS, re-sterilised by sintered glass filtration and stored at 4°C in 100ml amounts.

iii) Saline Trypsin Versene (STV)

This contained 0.01% of 1.250 trypsin (Difco, B152) and 0.01% Versene (Ethylenediaminetetraacetic acid) in PBS.

2.4 Bacterial strains

Most of the investigations with *Campylobacter sputorum* subspecies *mucosalis* were carried out with strain 253/72 (NCTC 11000). For comparative purposes parallel experiments were carried out on occasion with strains 722/75, 302/72 (NCTC 11,001), 140/76-220, 982/76 (NCTC 11419) or 512/77 (NCTC 11420).

Two strains of *Escherichia coli* one carrying the K88 antigen ($K88^+$) and a derivative which had spontaneously lost this character ($K88^-$) were used in the study of CSM attachment to pig brush borders.

Cultures were obtained from Dr G.H.K. Lawson, Department of Veterinary Pathology, University of Edinburgh and details are shown in Table 1.

Strain 253/72 was received as a plate culture which constituted the ninth sub-culture from original isolation, thereafter the strain was maintained by sub-culture at approximately ten day intervals. No differences in the behaviour of this strain in the infection of PK-15 cells were detected from the start to finish of this work.

Table 1

Bacterial strains used in investigations

Species	Strain	Serotype	Source
Campylobacter sputorum ss mucosalis	253/72 (NCTC 11000)	A - I	PIA
Campylobacter sputorum ss mucosalis	302/72 (NCTC 11001)	A - II	PIA
Campylobacter sputorum ss mucosalis	140/76-220	A - IV	PIA
Campylobacter sputorum ss mucosalis	722/75	A - V	PIA
Campylobacter sputorum ss mucosalis	982/76 (NCTC 11419)	B	PIA
Campylobacter sputorum ss mucosalis	512/77 (NCTC 11420)	C	PHE
Escherichia coli	114/72	0149:K91:K88ac	pig intestine
Escherichia coli		0149:K91	pig intestine

2.5 Routine cultivation and maintenance of bacteria

2.5.1 Campylobacter sputorum ss mucosalis

CBA plates were inoculated with CSM and placed in a McIntosh and Fildes anaerobic jar without a catalyst. The jar was evacuated to a negative pressure of 650mm of mercury, rechecked after 10 minutes and filled with hydrogen. Ten per cent of this atmosphere was removed and replaced with carbon dioxide prior to incubation at 37°C. Cultures were maintained in the hydrogen microaerophilic atmosphere and

subcultured at weekly intervals.

2.5.2 Escherichia coli

CBA plates inoculated with $K88^+$ and $K88^-$ were incubated at 37°C and 18°C respectively; strains were subcultured at weekly intervals.

2.5.3 Preparation of bacterial inocula

Twenty four- hours old surface growth of CSM or E. coli on CBA slope was removed by gentle washing with 10ml of prewarmed (37°C) MEM for infection of cell cultures or with PBS for tests of attachment to brush borders. The bacterial count of the infecting inoculum was determined by surface viable counts, vide infra.

2.5.4 Counting of bacterial suspension

Bacterial counts were carried out using a similar procedure to the surface viable count method described by Miles, Misra and Irwin (1938). Serial 10-fold dilutions were prepared from bacterial suspensions in sterile PBS. Using a calibrated sterile Pasteur pipette 0.02ml of each dilution was dropped on the surface of separate numbered sectors of two dry CBA plates. Plates were kept for 10 minutes at room temperature and then incubated as appropriate. The number of colonies in the highest dilution yielding growth was used to determine the number of bacteria present in the bacterial suspension.

2.5.5 Bacterial counts of cell cultures infected with CSM

Bacterial counts of supernatant fluids of infected cell monolayers were determined by surface viable counts vide supra.

2.5.6 Sources of CSM antisera

Hyperimmune sera were prepared in rabbits against viable cells of CSM strains 253/72 and 1258/78 (Lawson and Rowland, 1974). Each has an agglutinating titre of $\geq \frac{1}{1280}$ in whole cell agglutination tests with the homologous and heterologous strain.

Hyperimmune sera from rabbits immunised with bacteria extracted from PHE tissues were also used (Lawson et al, 1979).

All the antisera used were supplied by Dr Lawson.

2.6 Cell culture procedures

2.6.1 Growth and maintenance of cell cultures

Cells were cultured in 2ml-screw-capped vials, 6" x $\frac{5}{8}$" Pyrex test tubes, 4 oz medical flats, Brockway "Saniglass" cell culture bottles and one litre Roux flasks. The volume of the medium used in each type of container was 1, 1, 10, 50 and 100ml respectively and the cells were seeded at 10^5 cells per ml unless stated otherwise.

The cells were grown in Dulbecco's minimum essential medium (MEM) with 10% heat inactivated calf serum and for maintenance the serum was reduced by 1%. Antibiotics were not incorporated in growth or maintenance media unless so stated.

All cell cultures were subcultured every 7 days at which time the cells were detached from the glass with 10ml STV. The cells were pelleted at 200 x g, resuspended in growth medium, counted in a Neubauer counting chamber and diluted appropriately before seeding.

The monolayers were incubated for 3-4 days at 37°C and were refed with maintenance medium. Cell lines were routinely checked monthly for mycoplasma contamination by a method similar to that described by Hayflick (1965). Thus, 0.1ml of cell suspension was spread

on PPLO agar plates. These were incubated aerobically, anaerobically and hydrogen microaerophilically at 37°C for 7 days. Cultures suspected of mycoplasma or contamination were discarded.

2.6.2 Cell count determination

Trypan blue (0.2%) for determining the viability of cells was dissolved in 0.9% sodium chloride (Boyum, 1968). Cell counts were carried out using a haemocytometer (New improved Neubauer) according to the procedure recommended by Schalm et al (1975). Thus 0.1ml of a thoroughly mixed culture suspension was added to 0.1ml of 0.2% trypan blue in a bijou bottle and allowed to stand for 5 minutes. The mixture was then agitated and introduced into a haemocytometer. Unstained cells in 4 primary corner squares were counted to obtain the number of viable cells per ml.

2.7 Staining of adenomatous tissue and cell culture

2.7.1 Brucella differential staining

Smears of adenomatous suspensions of filtrates on glass slides were air-fixed, flooded with dilute carbol fuschin for 5 minutes and washed under slow-running tap water for 5 minutes. Smears were then decolorised with ½% acetic acid for 15 seconds, washed again under tap water for 5 minutes, counter-stained with methylene blue for 20 seconds, and then finally rinsed under tap water, air dried and examined by light microscopy.

2.7.2 Giemsa staining

Cell monolayers grown on glass coverslips were fixed in methanol, immersed in a 1:5 solution of Giemsa's stain in distilled water for 10 minutes and then differentiated in Giemsa buffer pH 6.8 for another

10 minutes.

Stained monolayers were then dehydrated and cleared through acetone, acetone-xylol (1:1) and xylol, mounted in Depex and examined by light microscopy.

2.8 Light microscopy

Unfixed monolayers were examined routinely by light microscopy using an Olympus inverted microscope. Monolayers stained by Giemsa's method, bacteriological smears, cell counts and autoradiographic preparations were examined using a Wild light microscope (Heerbrugg, Switzerland).

2.9 Immunofluorescent staining

Monolayer cultures on coverslips were washed 10 times in warm PBS, lightly blotted, fixed for 10 minutes in either ice cold acetone or formalin (demonstrate only extracellular organisms) and then air dried at room temperature for 15 minutes. Dried coverslips were either stained immediately or stored at -30°C in small tightly stoppered bijoux bottles containing silica gel. The staining technique was according to the standard indirect method (Nairn, 1976) using an appropriate dilution ($\frac{1}{20}$ in PBS) of rabbit anti-CSM serum (strains 253/72 or 1258/78, 'OH'-antiserum), or ($\frac{1}{80}$ in PBS) of rabbit anti-tissue extracted bacteria serum (RIΩS). The coverslips were flooded with diluted serum and incubated at 37°C in a moist chamber for 30 minutes, then rinsed twice in warm (37°C) PBS and thereafter washed continuously in PBS with gentle stirring with a magnetic stirrer at 37°C for 30 minutes. The coverslips were gently blotted to remove excess PBS then stained with an appropriate dilution ($\frac{1}{20}$ in PBS) of fluorescein labelled anti-rabbit

globulin prepared in sheep, incubated, rinsed and washed as before. The coverslips were mounted in glycerol-PBS buffer and examined in an Ortholux II photomicroscope (Ernst Leitz Wetzlar GmBH, Germany) by incident blue-light illumination from an ultra-violet light source (Wotan HBO 50 WA/C, Ploemepak 2 filter system).

2.10 Electron microscopy

2.10.1 Transmission electron microscopy

a) Preparation of Epoxy resin in lined culture dishes (Spurr, 1969)

Standard formulation

Vinyl cyclohexene dioxide (ERL 4206)	10.0 gm
Diglycidyl ether or polypropylene glycol (DER 736)	6.0 gm
Nonenyl succinic anhydride (NSA)	26.0 gm
Dimethyl aminoethanol (S-1)	0.4 gm

The appropriate weight of each of the first three liquid chemicals was successively pipetted directly into a pre-weighed container on an Oertling balance, mixed well, S-1 added and mixed again. The resin was then dispensed in 4ml amounts into vented tissue culture petri dishes 50mm x 15mm (NUNC International, Denmark) and incubated at 60°C for 16 hours after which time the resin solidifies.

b) Growth of cells on epoxy resin surfaces

The surface of resin and the petri dishes that were to be in contact with culture medium were sterilised by irradiation with a U/V lamp (Desaga, Heidelberg) at 366nm for 1 hour in a culture hood.

MEM was added to dishes and incubated overnight at 37°C. Dishes were drained and rinsed with fresh culture medium before

seeding with cells. To each dish 3ml of cell suspension in MEM were added at a concentration of 1 x 10^7 cells per ml. The dishes with their contents were then incubated in a McIntosh and Fildes anaerobic jar in the presence of 10% carbon dioxide at 37°C.

c) Fixing of cells

Solutions used:

i) Cacodylate stock solution

This was prepared by dissolving 1 litre of distilled water, 0.86gm of Magnesium acetate ($(CH3COO)_2Mg .4H_{20}$) and 42.8gm of sodium cacodylate and the pH adjusted to 7.0 with hydrochloric acid. The solution was then filtered through sintered glass and stored at -30°C in 100ml amounts until needed.

ii) Fixative

The fixative was prepared by adding 50ml of the cacodylate stock solution to 12ml vacuum-distilled 25% glutaraldehyde (Agar Aids, Stansted, Essex, England) and making the volume up to 100ml with distilled water. The fixative was stored in a dark bottle at 4°C and used within 2 weeks of preparation.

iii) Veronal acetate stock solution

This stock solution was prepared by dissolving 1.47gm of sodium barbitone and 0.585gm of anhydrous sodium acetate in 500ml of distilled water. This was filtered through sintered glass and stored at -30°C in 100ml amounts until needed.

iv) Osmium tetroxide post-fixative

The Osmium tetroxide post-fixative was prepared by dissolving 0.68gm of sucrose in a mixture of 5ml 2% Osmium tetroxide, 2ml veronal acetate stock solution, 2ml 0.1N hydrochloric acid and 1ml distilled water. This was stored at 4°C and used within 3 weeks of

preparation.

v) Cacodylate-sucrose rinse buffer

This was prepared by dissolving 5gm sucrose in 50ml cacodylate stock solution then diluting to 100ml with distilled water. The solution was always made up and used fresh.

vi) Veronal acetate-acid rinse buffer

This rinse buffer was prepared by mixing 10ml veronal acetate stock solution with 24ml 0.1N hydrochloric acid and then making it up to 100ml with distilled water. The buffer was always made up and used fresh.

vii) Fixation

Cell were fixed by rinsing the cultures with sterile PBS at room temperature, discarding the fluid and then covering the monolayers with 3ml glutaraldehyde fixative (ii). The fixation process was carried out for 30 minutes at 4°C, when the cell monolayer was rinsed twice with cacodylate-sucrose rinse buffer (v) and afterwards allowed to stand in two changes of fresh buffer, each for periods of 15 minutes at 4°C.

At the end of the rinsing period the fluid was drained off and the cell monolayers were overlaid with 1ml Osmium tetroxide post-fixative (iv) in a fume cupboard. After 30 minutes the cells were finally rinsed in several changes of veronal-acetate acid buffer (vi) in the manner described previously.

d) Dehydration and polymerisation of cells

Dehydration was carried out with graded concentrations of ethanol. After the monolayer had been rinsed in veronal acetate buffer (vi) the fluid was drained and the monolayer covered with 50% ethanol for 20 minutes. This was replaced successively with

70%, 90% and 100% ethanol, each for 20 minutes.

The dehydrated cell monolayer was drained, then overlayered with freshly prepared epoxy resin which was left uncovered at room temperature for 24 hours. The resin was discarded and finally replaced with fresh resin after which the petri dish was held at 60°C for 16 hours to allow the resin to solidify.

e) Cutting of sections and viewing of cells

The embedded cells were cut at approximately 60nm thick on an OM4 microtome (Reichert, Austria) with a diamond knife, stained with saturated uranyl acetate in 50% ethanol and lead citrate (Reynolds, 1963) and examined in an electron microscope (Philips, Nederland, Br Hoofdgroep, PPS Postbus 90050, 5600PB Einhoven) at 100kv.

2.10.2 Scanning electron microscopy

Cells for scanning electron microscopy were grown as monolayers on glass coverslips. The cells were fixed and dehydrated in the same manner as those for transmission electron microscopy. After dehydration the coverslips were mounted on aluminium stubs and coated with gold on an Emscope spotter coater Sc 500 (Emscope, Kent, England). These were examined in a Philips 505 scanning electron microscope (Philips, Nederland, Postbus 90050, Einhoven) at 30kv.

CHAPTER 3.

Chapter 3

Infection of pig kidney cells (PK15) with "cultured" *Campylobacter sputorum* subspecies *mucosalis* (CSM-C)†

† "Cultured CSM" refers to CSM grown in cell free media. In later sections specific reference will be made to CSM derived from pig tissue CSM-T or from tissue culture CSM-TC.

Chapter 3

3. Infection of PK15 cells with "cultured" Campylobacter sputorum subspecies mucosalis

3.1 General Introduction

Campylobacter sputorum subspecies mucosalis was first isolated from clinical cases of PIA by Lawson and Rowland (1974), and subsequent studies by these workers have contributed to our knowledge of this organism and its association with enteric disorders in swine.

In recent years cell culture systems have been used increasingly to study the pathogenic mechanisms of microorganisms. Unfortunately, information is very limited on the growth and development of bacteria in tissue culture and, consequently, the response of host cells to bacterial infections is not well understood. This deficiency is partly because the study of bacterial host cell interaction in tissue culture is made difficult by the extracellular bacterial growth which may take place and which is often associated with or responsible for the degenerative changes in the infected monolayer. For this and other reasons much of the early successful research in this field (Fell and Brieger, 1947; Hanks, 1956) involved slow-growing bacteria such as mycobacteria which are phagocytosed by the cells before they are able to multiply in the extracellular fluid.

Many bacterial intracellular parasites primarily infect the phagocytic cells for example, macrophages or polymorphonuclear leucocytes and consequently, the epithelial or penetration stage has been of less interest or of less importance. Infection of cell

lines derived from epithelium therefore may have little relevance to much of the disease process and most studies have tended to concentrate on phagocytic cell infection. In addition, many important bacterial infections are not intracellular at any stage. In contrast, in virology where extracellular growth of viable virus particles is not possible, considerable progress has been made in understanding the growth and development of viruses in vitro and the mechanisms involved in the pathogenesis of viral infections.

Many workers have attempted to exclude extracellular multiplication of bacteria by including antibiotics in cell culture media (Kihlström, 1977; Devenish and Schiemann, 1981; Mapother and Songer, 1984). This may however only modify extracellular growth and is likely to introduce new problems of interpretation because the extent to which antibiotics are excluded from the cell cytoplasm is not always clear and their presence may interfere with intracellular survival and the multiplication or recovery of bacteria. A further problem inherent in the use of antibiotics is that bacteria may develop resistance to the antibiotics and multiply thereafter. Whilst it is easy to avoid errors due to the acquisition of complete resistance, small changes in the minimum inhibitory concentration of the antibiotics for the bacterial strain may be less easy to interpret.

In the study of cell cultured infection with CSM-C this problem does not arise as antibiotics are not required to be added to the medium in order to control extracellular growth. This is because CSM does not multiply in tissue culture fluid that does not contain cells that permit attachment of the bacteria (Rajasekhar, 1981) provided that the fluid of the media is exposed to oxygen at atmospheric pressures.

Rajasekhar (1981) reported that CSM not only attached in greater numbers to PK15 or PKpi cells than to any other cells that he investigated but was phagocytosed by these cells and thereafter included a number of unusual cytopathic effects. The majority of these experiments were carried out with a "standard" infecting dose and no information is available on the effect of weight of inoculum. In order to confirm Rajasekhar's results and to allow the author to become familiar with the cytopathic changes in PK15 and to examine the effect of variations in the infective dose of CSM or the concentration of tissue culture cells, the following experiments were carried out.

3.2 Cytopathic changes produced by exposing PK15 cells to a "standard" CSM infective dose.

3.2.1 Introduction

A fixed concentration of PK15 cells were infected with a "standard" CSM infecting dose. The cytopathic changes produced by this infection were recorded and used for comparison with the effects produced at different cell and CSM concentrations. Bacterial growth in the cell culture was monitored by sampling the supernatant fluid at appropriate times.

Staining methods were used to demonstrate the interaction of CSM with PK15 cells. Unfortunately, Giemsa staining which is the least tedious of the four available methods is an unreliable method of demonstrating intracellular CSM (Rajasekhar, 1981). Intracellular organisms were demonstrated by transmission electron microscopy and immunofluorescence staining of acetone-fixed coverslips, while extracellular organisms were shown by scanning electron microscopy

and immunofluorescence staining of formalin-fixed coverslips.

3.2.2 Materials and Methods

i) Growth of CSM on CBA slopes

CBA slopes in 1 ounce McCartney bottles were inoculated with CSM and the bottles stoppered with cotton wool. The bottles and contents were incubated for 24 hours as described in 2.5.1.

ii) Growth of cells on coverslips and resin surfaces

PK15 cells (Gibco (Biocult) Europe Ltd., Paisley, Scotland) were grown on coverslips in test tubes as described in 2.6.1 and on resin surfaces as in 2.10.1.

iii) Infection of cells with CSM

A 24 hour-old surface growth of CSM strain 253/72 on CBA slope was removed by gentle washing with 10ml of pre-warmed (37°C) MEM. The suspension of bacteria thus obtained generally contained between 10 $\log_{10}$ - 11 $\log_{10}$ organisms per ml; as determined by surface viable counts (Miles et al, 1938). Infective doses are listed in the tables (2 and 3a) relevant to specific experiments.

Cells grown for 24 hours on coverslips in tubes or on resin in petri dishes were overlaid with 1 ml or 3 ml of the suspension of CSM in MEM respectively and incubated at 37°C. Each day surface viable counts of the supernatant fluid from infected coverslip cultures were performed on duplicate CBA plates. Coverslips were removed after 1 hour and thereafter daily for ten days and processed for scanning electron microscopy, immunofluorescence and Giemsa

staining by methods described in 2.10.2, 2.9 and 2.7.2 respectively. Uninfected cells were also processed as above and used as controls. Cells grown in resin were fixed each day post infection and processed for transmission electron microscopy as described in 2.10.1. Infected and control cells were refed after every 3 days with maintenance medium. This was achieved by draining the supernatant fluid and immediately replacing it with 1 ml or 3 ml of maintenance medium.

3.2.3 Results

i) One hour post infection

Immunofluorescence, Giemsa stained preparations, and scanning electron micrographs of PK15 cells exposed to CSM for one hour showed adherent bacteria on the surface of about 95% of the cells with individual cells showing different degrees of bacterial attachment (from less than 10 to uncountable numbers per cell) where bacteria were frequently clustered at the edge of the cytoplasm. This attachment was particularly marked at the periphery of the cell, fewer organisms were adherent to other parts of the cell surface (Fig. 1) Scanning electron micrographs showed that organisms were in close contact with, and aligned horizontally along, the surface of the cell (Fig.2). There was no evidence of bacterial attachment to cells by means of the flagella. At this stage of infection there was no sign of phagocytoses or penetration of CSM into cells.

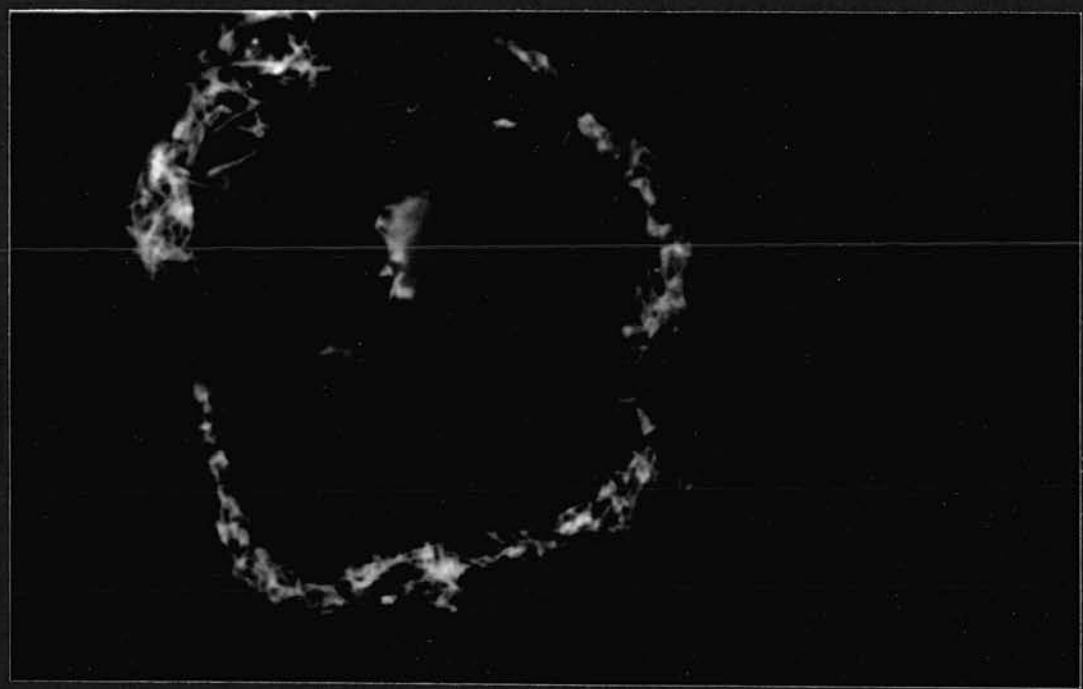
There were no visible changes either of the surface of the cells or in the cytoplasm of stained preparations. There had been a decrease in the number of viable CSM recovered from the supernatant fluid of infected PK15 cells in comparison with the numbers present immediately after inoculation (Table 2).

Fig. 1. PK15 cells of 24 hour-old monolayer 1 hour after infection with CSM showing extensive bacterial attachment to the periphery of the cell. Acetone fixation, immunofluorescence staining with rabbit anti-CSM serum and sheep anti-rabbit (FITC) conjugate.

(x 284)

Fig. 2. Scanning electron micrograph of 24 hour-old PK15 cells 1 hour after infection with CSM showing bacteria irregularly attached at cell periphery.

(x 41175)



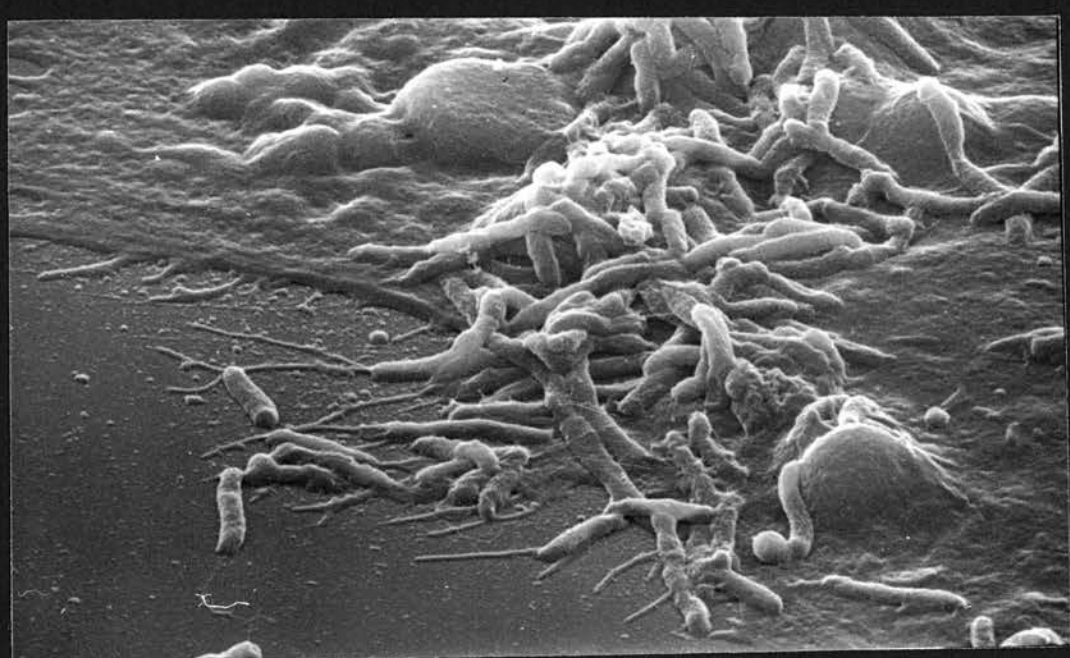


Table 2: Cytopathic changes produced in PK15 cells infected by CSM and the number of CSM isolated from the supernatant fluids

Time after infection in Hours (h) or Days (d)	Cytopathic changes (CPE) and intracellular bacterial parasitism		Bacterial count in supernatant as organisms $\log_{10}$ per ml
0h	0		10.05
1h	0	(95)	9.65
1d	0	(95)	6.06
2d	1[a]	ND	6.13
3d	2	(95)	6.00
4d	2	ND	5.48
5d	3	(95)	5.24
6d	3	ND	5.00
7d	4	(97)	4.70
8d	4	ND	4.68
9d	5	(98)	3.10
10d	5	ND	2.40
11d	6	ND	NR

ND = Not examined

NR = No organisms recovered

0 = No CPE

a = Increasing severity of CPE, 6 - complete cell detachment from coverslip

() = Numbers in brackets indicating the percentage of cells showing intracellular fluorescence.

ii) One day post infection

Examination of Giemsa-stained and unstained monolayers of infected cells showed that cell growth and monolayer-formation were not affected at this time by infection with CSM. There were no unusual morphological changes at this stage and organisms were not seen on cell surfaces. This later observation was confirmed by immunofluorescent staining of formalin-fixed coverslips and scanning electron microscopy (Fig. 3) which showed an absence of bacterial antigen or bacterial forms respectively. There was a dramatic reduction in the numbers of viable infecting bacteria in the supernatant fluid and all subsequent examinations showed a steady progressive fall in bacterial recovery up to the 10th day (Table 2).

iii) Two days post infection

After two days of infection, gaps visible microscopically had developed in the monolayer. Immunofluorescent staining of acetone-fixed infected cells showed small amounts of intracytoplasmic round particulate antigen which formalin-fixed cells did not demonstrate.

There was rounding up of some 10% of the cells and these abnormal cells showed a granularity of the cytoplasm. A similar percentage of cells had enlarged nuclei and a few cells had started to fuse (Fig. 4a). Some cells had small vacuoles in the cytoplasm situated towards the cell periphery. All infected cells at this stage remained attached to the glass. Uninfected control cells did not show any cellular changes (Fig. 4b).

Fig. 3. Scanning electron micrograph of PK15 monolayer 24 hours after infection with CSM. Organisms are absent from the cell surface.

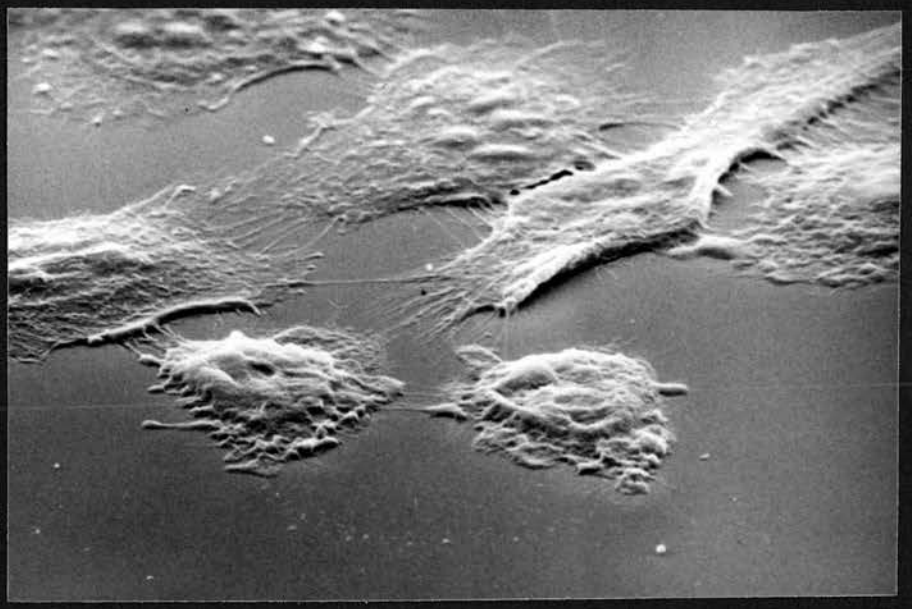
(x 6120)

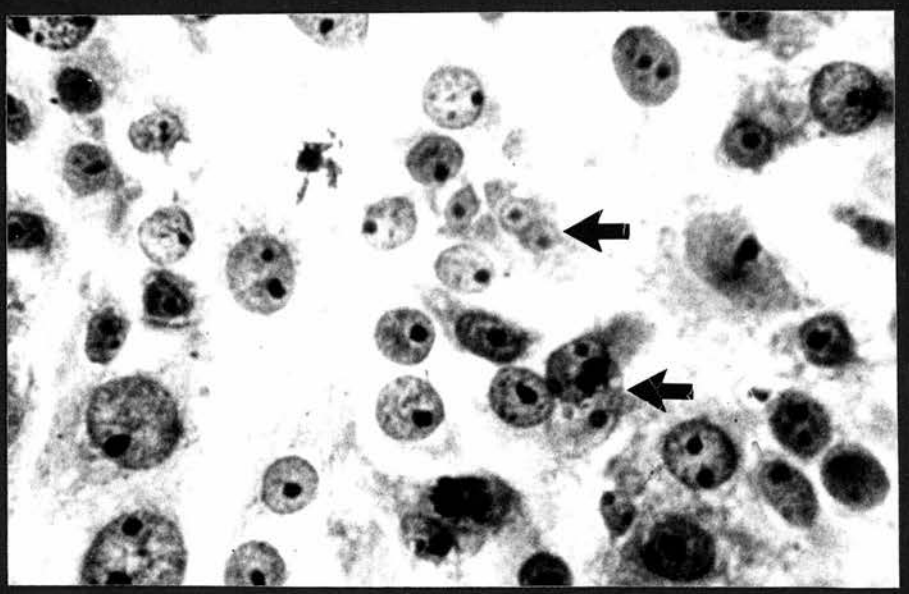
Fig. 4a. PK15 monolayer 48 hours after infection with CSM. Some cells contain enlarged nuclei. Note cells at early stage of fusion (arrowed).

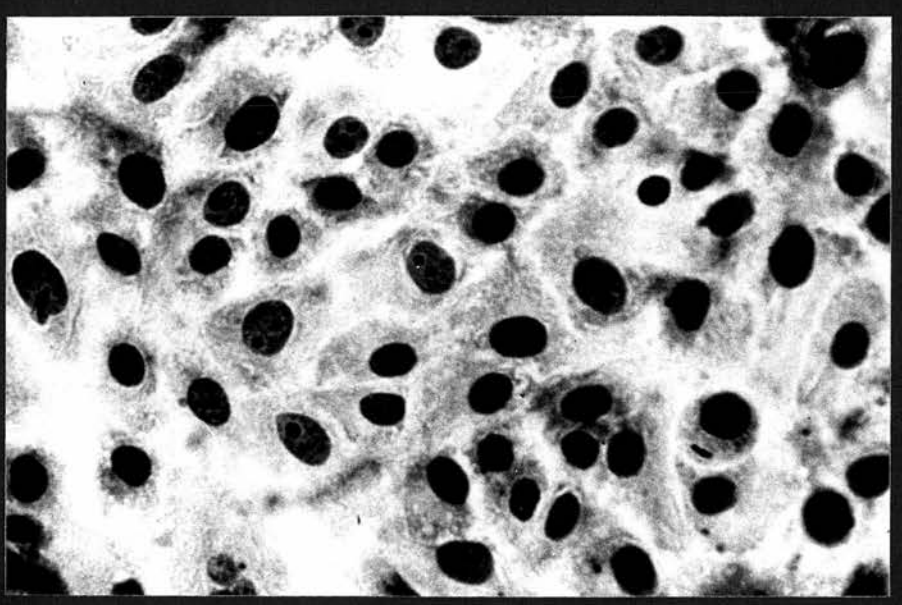
Giemsa stain (x 113)

Fig. 4b. Uninfected PK15 monolayer, 48 hours after seeding.

Giemsa stain (x 113)







iv) Three and four days post infection

There was progressive destruction of cell monolayer with increased cellular granularity, vacuolation in the depths of the cytoplasm and rounding up of cells ending in cell detachment from the glass. The supernatant fluid was cloudy, acidic and contained clumps of abnormal cells. About 30% of the remaining cells of the monolayer were swollen with enlarged nuclei and more cells were rounding up (Fig. 5a). Uninfected control cells did not show any changes (Fig. 5b).

Scanning electron micrographs showed the presence of cell-surface blebs pits and crevices which indicated cell injury (Fig. 6).

Transmission electron micrographs showed that in most specimens, organisms were present in phagosomes and the membranes lining these vacuoles were intact. However, many bacteria were also present on the cytoplasm apparently not surrounded by a limiting membrane.

Immunofluorescence staining of acetone-fixed monolayers showed an increased amount of round particulate antigen scattered in the cytoplasm but not in the nucleus.

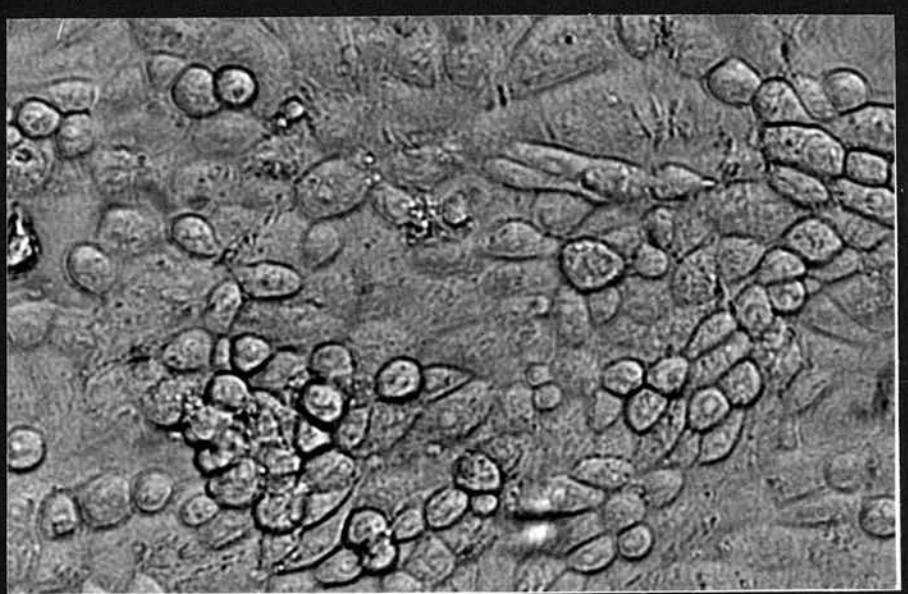
v) Five to nine days post infection

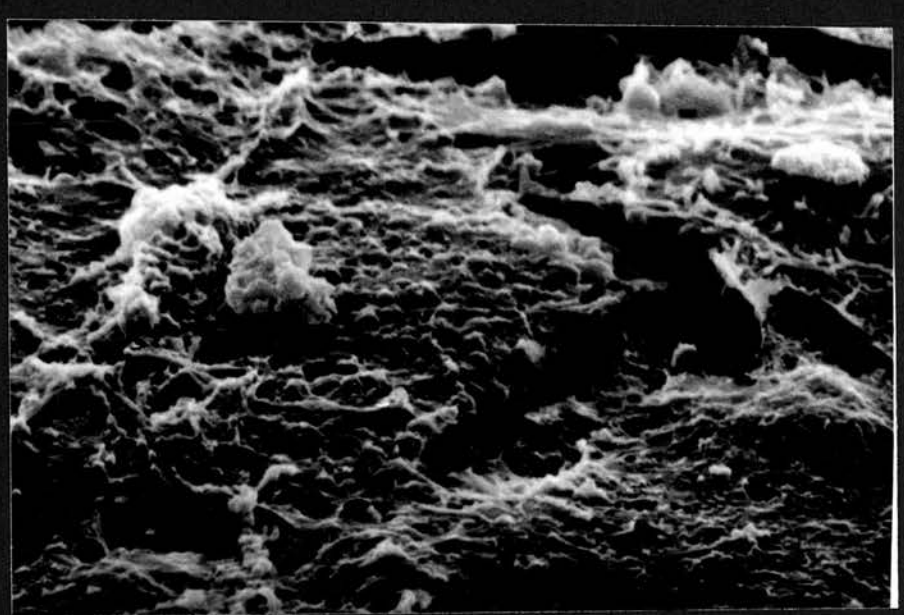
There was progressive decrease in the number of CSM isolated from the supernatant fluid. The fluid was cloudy, acidic and always contained clumps of abnormal cells. The monolayers principally contained cells with enormously enlarged cytoplasm and nuclei. These abnormal cells were quite distinct from a small number of apparently normal cells similar to uninfected control cells, these normal cells comprised some 5% of the cells of the infected monolayer. There had been progressive destruction of the cell monolayer with abnormal, enlarged, multinucleated syncytia and extensively vacuolated cells

Fig. 5a. Unstained monolayer of PK15 cells 4 days after infection with CSM showing numerous abnormal cells with swollen cytoplasms. A number of cells rounding up are apparent.
(x 113)

Fig. 5b. Unstained uninfected 4 days old monolayer of PK15 cells. The cells form a confluent monolayer at this time.
(x 113)

Fig. 6. Scanning electron micrograph of PK15 cells 4 days after infection with CSM. The cell surfaces show pits and crevices. Note the absence of organisms on the cell surface.
(x 19800)





all remaining in patches on the coverslips. These polykaryons now contained multiple nuclei of varying sizes. The vacuoles varied greatly in sizes and numbers and replaced the entire cytoplasm in some cells (Fig. 9). At this stage these altered cells contained coccal forms which fluoresced with CSM antiserum in immunofluorescent stained preparations (Fig. 10).

Transmission electron micrographs showed that some bacteria were still lying free within the cytoplasm and appeared to retain their usual morphology, although, degenerate bacterial cells were also present (Figs. 7,8). Bacteria lying within phagosomes has undergone extensive degeneration and the contents of these vacuoles mostly consisted of "ghost cells" presumably representing degenerate forms of CSM. The origin of the residual structure of the "ghost cells" is not clear but their hollow ring-like appearance seemed to represent altered but intact cell walls of CSM devoid of normal cytoplasmic material (Figs. 7, 8). An unusual organelle was present in some cells, often in the perinuclear area, these consisted of a membrane bound structure with irregular finger-like granular processes protruding into the lumen of the unstained vacuole(Fig. 8). Possibly these represent damaged bloated mitochondria with dilated outer mitochondrial spaces. No other visible changes were evident in the other cell structures, although, there were lipid droplets distributed in the cytoplasm.

vi) Ten and eleven days post infection

Cells remained in isolated patches and were abnormal with grossly enlarged cytoplasm and nuclei. The supernatant fluid was cloudy, and contained large clumps of abnormal cells. After 11 days there was total detachment of cells from the coverslips and at this time CSM could no longer be recovered from the culture fluids.

Fig. 7. Transmission electron micrograph of PK15 cells infected with CSM 5 days previously. Bacteria (A) free in the cytoplasm and within the phagosomes (arrowed) at various stages of degeneration resulting in "ghost cells" of bacteria.

(x 31250)

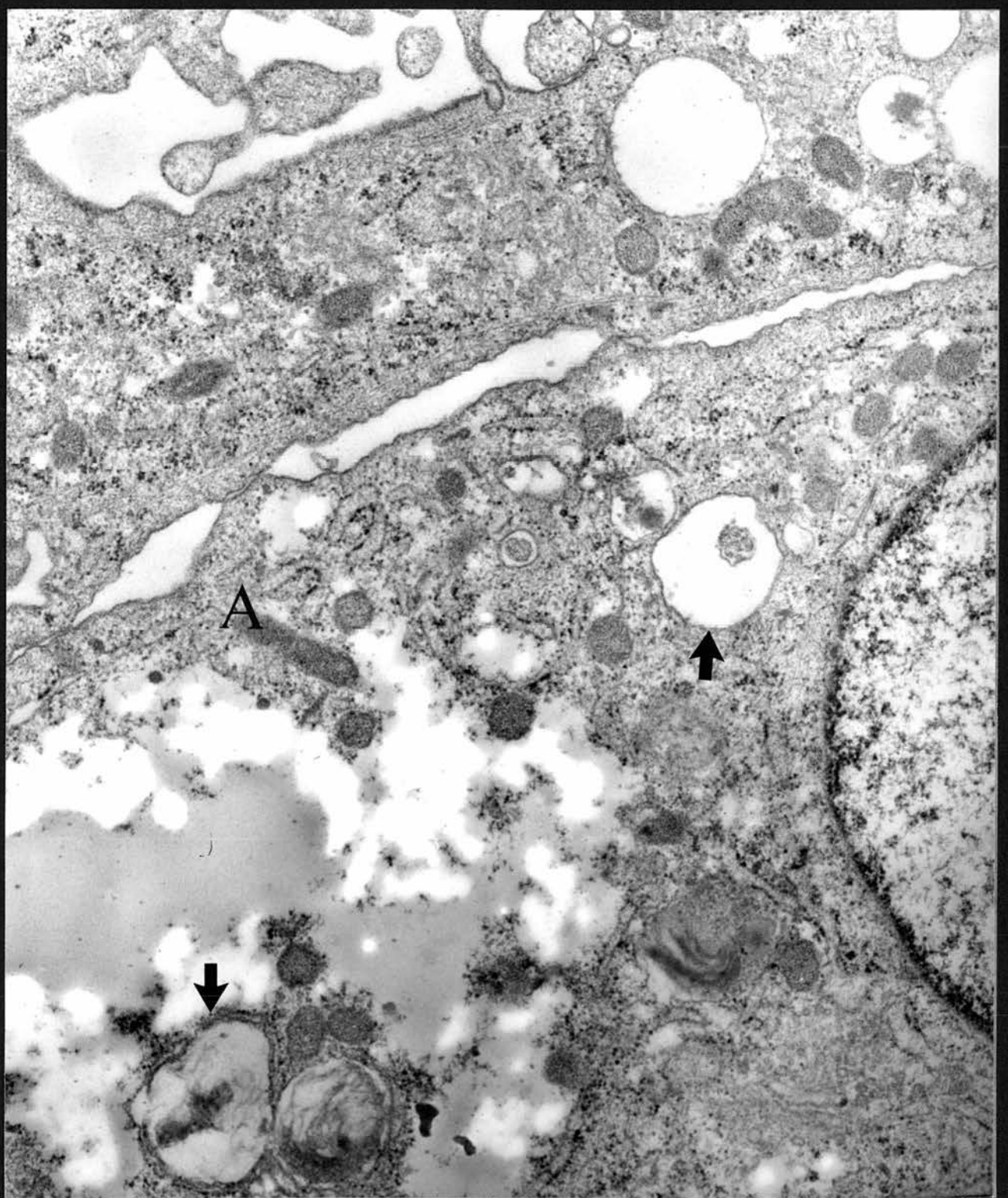

A

Fig. 8. Transmission electron micrograph of PK15 cells after 5 days of infection with CSM. Bacteria (A) within the phagosomes and lying free, (B) in the cytoplasm not surrounded by a host membrane. Note an enlarged cell organelle (arrowed) showing a dilated vascular space containing finger-like processes.

(x 15000)

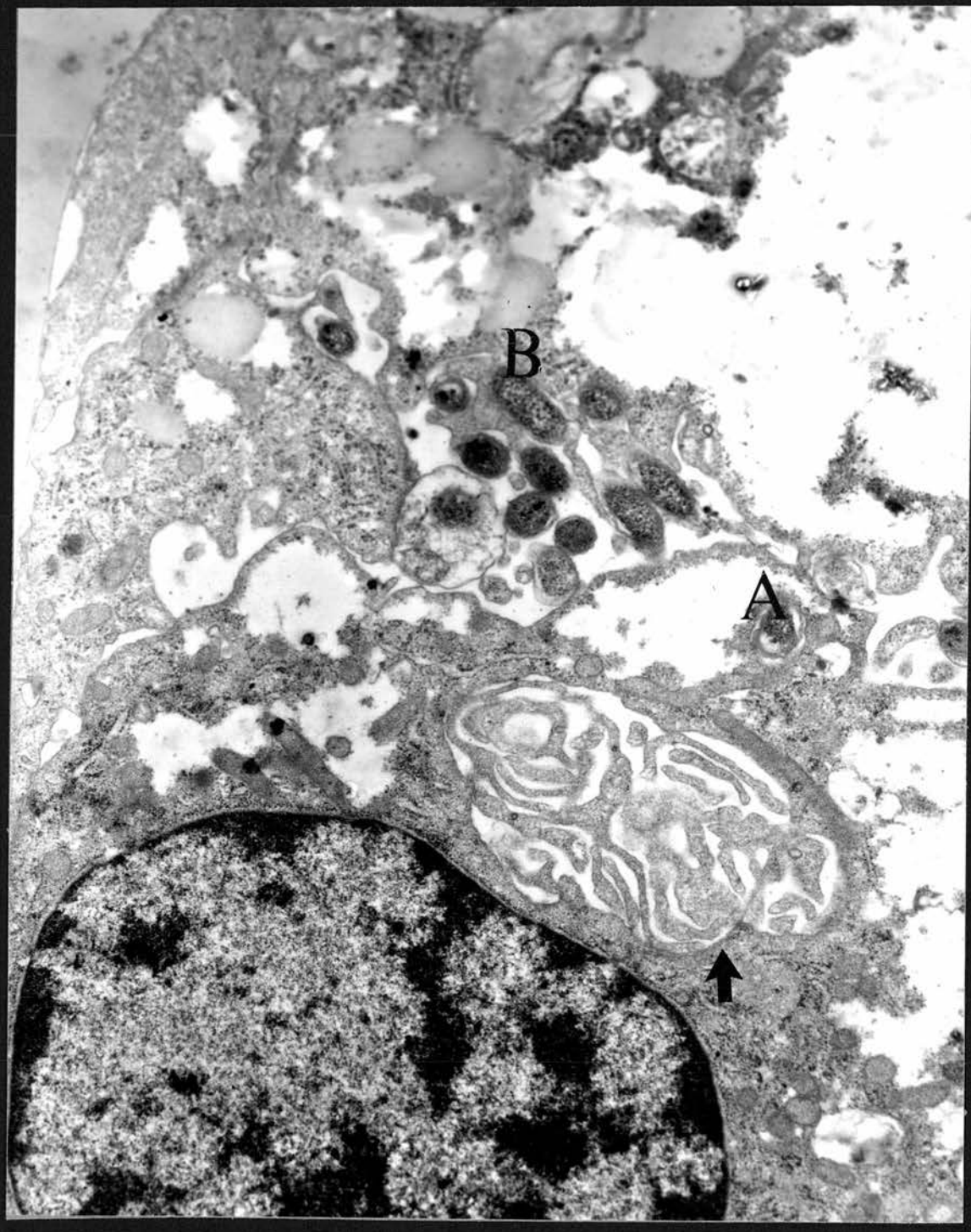
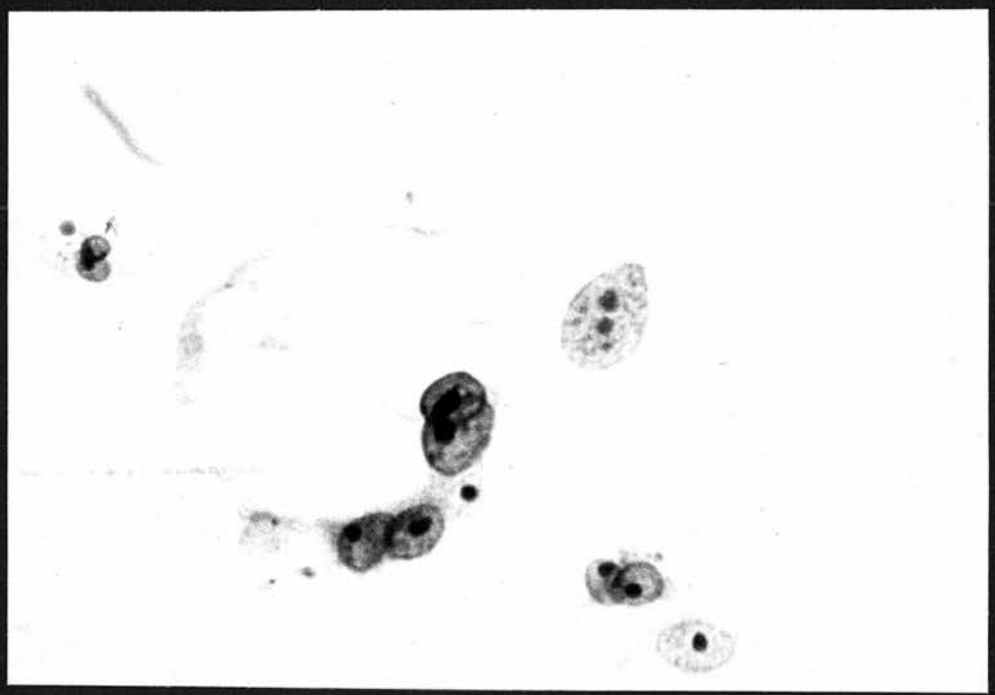

B
A

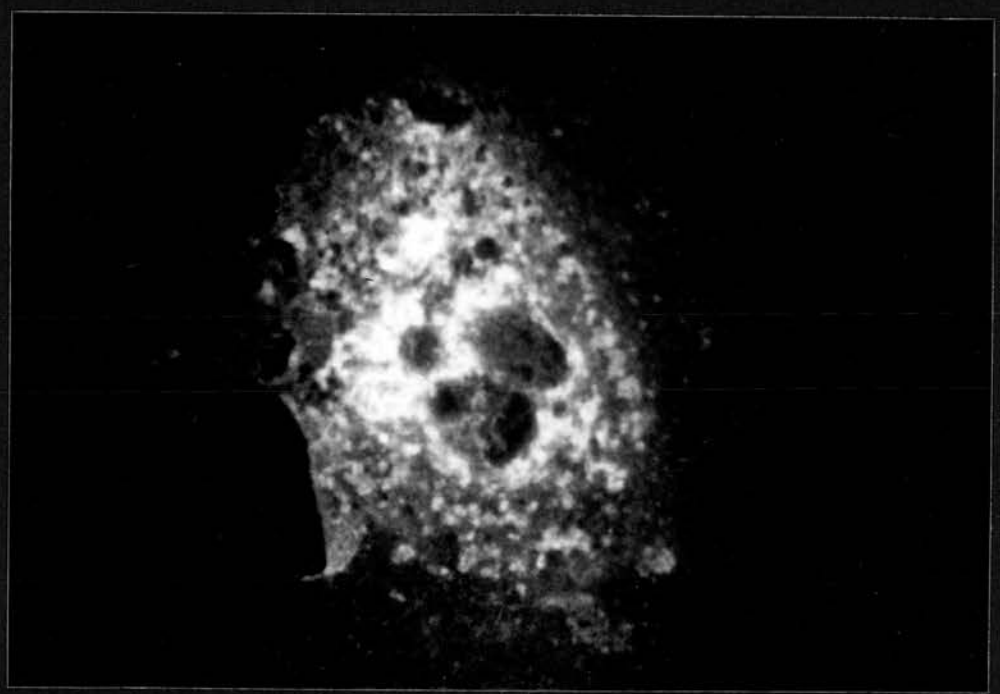
Fig. 9. PK15 cells 9 days after infection with CSM. Most of the cells of the monolayer have been destroyed and detached from the coverslip. The CPE is characterised by massive vacuolation of the cytoplasm and multiple nuclei.

Giemsa stain (x 113)

Fig. 10. PK15 cells 9 days after infection with CSM showing extensive intracytoplasmic fluorescing particles, the cell nuclei are not involved. The cell outline is irregular. Acetone fixation, immunofluorescence staining with rabbit anti-CSM serum and sheep anti-rabbit (FITC) conjugate.

(x 284)





3.2.4 Comments

Cytopathic changes produced by CSM in PK15 cells were characterised by rounding up, cytoplasmic granularity, fusion of cells and detachment from the glass leaving 'holes' in the residual cell monolayer.

Cytopathic changes were evident after 2 days of infection and progressed with increased enlargement of both cytoplasm and nuclei, cellular vacuolation, polykaryon formation which by the 11th day terminated in total detachment of cells from coverslips. These changes may be also a result of the increased and prolonged exposure of cells to acid conditions brought about by the metabolic products of infected cells.

There was a rapid drop of the number of viable CSM in the supernatant fluid after 1 day of infection after which there was a more gradual continued decrease in number until no CSM could be recovered from the fluid 11 days after infection at the time of final disruption of the monolayer.

3.3 The effects of exposing PK15 cells to different concentrations of CSM

3.3.1 Introduction

Infection of preformed monolayers of PK15 cells with a "standard" CSM infecting dose produced cellular abnormalities. To examine whether these changes were infecting-dose related, cells in monolayer culture were exposed to varying concentrations of CSM.

3.3.2 Materials and Methods

A 24 hour-old surface growth of CSM strain 253/72 on CBA slope was removed by gentle washing with 10 ml prewarmed (37°C) MEM. From this, two dilutions of CSM were made in warm MEM and the respective concentrations of each dilution determined by surface viable counts.

One ml of each dilution of CSM was separately used to infect 24 hour-old PK15 cells grown on coverslips. Coverslips were removed daily, fixed appropriately and stained by Giemsa's method and by immunofluorescence for CSM. Viable counts of CSM in supernatant fluid were carried out daily.

3.3.3 Results

At a concentration of 5.26 $\log_{10}$ organisms per ml, CSM did not produce any visible cytopathic changes in PK15 cells in the 13 days after exposure to infection, and infected cells remained similar to uninfected PK15 cells. About 11% of the cells showed small amounts of intracytoplasmic fluorescence of acetone fixed coverslips after 1 day of infection (Table 3a). Bacteria were not recovered from the supernatant fluid after 3 days (Table 3b).

At a concentration of 8.26 $\log_{10}$ organisms per ml, there was no visible effect after 1 and 2 days, but by the 3rd day cells had started to round up, microscopic 'holes' appeared on the monolayer marking the beginning of destruction of the monolayer. This destruction progressed with a comparable series of cellular abnormalities to that already described until the 13th day when there was total detachment of cells from the coverslips (Table 3a).

At CSM concentration of 11.26 $\log_{10}$ organisms per ml, the cytopathic changes produced were similar to those of 3.2.3 with the

Table 3a: Effects of exposure of PK15 cells to varying concentrations of CSM

Time after infection in days	Uninfected PK cells	Bacterial counts of infective dose as organisms $\log_{10}$ per ml					
		11.26		8.26		5.26	
1	0	0	(95)	0	(92)	0	(11)
2	0	1[a]	ND	0	ND	0	ND
3	0	2	(95)	1	(92)	0	(8)
4	0	2	ND	2	ND	0	ND
5	0	3	(95)	2	(92)	0	(5)
6	0	3	ND	3	ND	0	ND
7	0	4	(97)	3	ND	0	ND
8	0	4	ND	4	(95)	0	(4)
9	0	5	(98)	4	ND	0	ND
10	0	5	ND	5	ND	0	ND
11	0	6	ND	5	(98)	0	(0)
12	0	6	ND	5	ND	0	ND
13	0	6	ND	6	ND	0	ND

ND = Not examined

0 = No CPE

a = Increasing severity of CPE
1 - detectable CPE, 6 - Complete cell detachment from coverslip

( ) = Numbers in brackets indicating the percentage of cells showing intracellular fluorescence.

Table 3b: Number of CSM in the supernatant fluids of PK15 cells infected with varying concentrations of the organisms

Time after infection in days	Bacterial count as organisms $\log_{10}$ per ml		
	11.26*	8.26*	5.26*
1	6.54+	4.60+	3.44+
2	6.68	4.70	3.51
3	6.24	4.54	2.88
4	5.54	4.35	NR
6	4.70	3.60	NR
8	3.88	3.18	NR
9	3.54	2.40	NR
10	2.88	NR	NR
11	NR	NR	NR

NR = No organisms recovered

* = Counts of infective doses

\+ = Counts of supernatant fluid

total detachment of cells occurring after 11 days (Table 3a).

3.3.4 Comments

Cytopathic changes in PK15 cells were produced with an infecting dose of CSM of 8.26 $\log_{10}$ organisms per ml, above this level of infection cytopathic changes were produced sooner whilst at 5.26 $\log_{10}$ organisms per ml no cytopathic changes were produced. The approximate bacteria/cell ratio is 10^6:1 at CSM concentration of 11.26 $\log_{10}$ organisms per ml, 10^3:1 at 8.26 $\log_{10}$ organisms per ml and 1:1 at 5.26 $\log_{10}$ per ml. From the above results it appears that for progressive cytopathic infection to be produced in the cells the bacteria/cell ratio should be of the order of 1000:1, and lower levels may not achieve this effect.

The approximate bacteria to cell ratios were calculated from the number of cells seeded and the infective dose of bacteria. Since multiplication of cells would take place before infection there is likely to be a real small reduction in the bacteria/cell ratio in all instances. At CSM concentrations of 5.26 $\log_{10}$ organisms per ml, only an organism is available to attach to each cell and it is likely that there are insufficient bacteria to infect all cells in the population. After 3 days CSM was not recovered from the supernatant fluid, but a few of the cells showed fluorescence up to 8 days after infection. Organisms that did not attach to cells probably died in the fluid since it does not support growth of CSM and those that entered the cells appeared to be destroyed by the protective mechanisms of the cells.

3.4 The effects of different cell concentrations on the cytopathic changes brought about by CSM

3.4.1 Introduction

The cytopathic changes induced in PK15 cells by exposure to CSM has been shown to be related to the infecting dose. To examine whether the concentration of cells exposed to infection has any effect on the development of cytopathic changes, monolayers produced from different concentrations of PK15 cells were infected with a "standard" CSM infecting dose.

3.4.2 Materials and Methods

PK15 cells were grown on coverslips at initial seeded concentrations of 10^{11}, 10^{8}, 10^{5}, and 10^{3} cells in 1 ml. After 24 hours each cell monolayer was infected with 1 ml of 24 hour-old CSM culture suspended in warm MEM. Each monolayer was exposed to 10.09 $\log_{10}$ organisms. The infected cells were incubated at 37°C and coverslips were removed daily, and stained by immunofluorescence and Giemsa's methods. Uninfected cells of each concentration were used as controls.

3.4.3 Results

Cells grown from an initial concentration of 10^{3} cells per ml never formed a confluent monolayer and only grew in islands until the cells became totally detached from the glass 8 days after infection. At concentrations of 10^{5}, 10^{8} and 10^{11} cells per ml, cytopathic changes were evident after 2, 3 and 4 days and cells were completely detached from coverslips after 11, 12 and 14 days respectively (Table 4). Cytopathic changes produced in these cells were similar to those

Table 4: Effect of cell concentration on the production of cytopathic changes by CSM

Time after infection in days	Number of cells per ml.							
	10^3		10^5		10^8		10^{11}	
1	0	(98)	0	(95)	0	(93)	0	(90)
2	1[a]	ND	1	ND	0	ND	0	ND
3	2	(98)	2	(95)	1	(93)	0	(90)
4	3	ND	2	ND	1	ND	1	ND
5	4	ND	3	(95)	2	(93)	1	(90)
6	5	(99)	3	ND	2	ND	2	ND
7	5	ND	4	(97)	3	(96)	2	(92)
8	6	ND	4	ND	4	ND	3	ND
9			5	(98)	4	ND	4	(95)
10			5	ND	5	(98)	4	ND
11			6	ND	5	ND	5	(98)
12					6	ND	5	ND
13							5	ND
14							6	ND

ND = Not examined

0 = No CPE

a = Increasing severity of CPE
1 - Detectable CPE, 6 - Complete detachment from coverslip

( ) = Numbers in brackets indicating the percentage of cells showing intracellular fluorescence.

described previously.

3.4.4 Comments

A decrease in the concentration of cells exposed to a "standard" inoculum of CSM affected the rate of development of cytopathic changes. The higher the concentration of cells used to form the monolayer the longer it took for the CPE to develop and for the cells to become completely detached from coverslips.

3.5 Discussion

During the first 24 hours post infection there was a marked reduction in the number of viable bacteria in the supernatant fluids of infected monolayers which was followed by a further gradual decrease until CSM could no longer be isolated from the supernatant fluids after the 11th day (<50 organisms/ml).

CSM does not survive in tissue culture media in small volumes exposed to atmospheric oxygen tensions at 37°C, so organisms that do not attach may not survive while those that attach are phagocytosed by the cells. These two mechanisms are probably responsible for the initial drop in viable CSM in supernatant fluid. The origin of the viable CSM recovered from the supernatant fluids thereafter is not completely clear. The progressive degeneration of infected cells appears to be responsible for the daily recovery of CSM and viable bacteria may be present in detached but still intact host cells. When cellular destruction is extensive the organisms rapidly die. Cells seem to provide some essential metabolites or necessary gaseous environment for the growth and survival of organisms.

The identity of the perinuclear organelle seen between 5 and 9 days is unclear and clarification would require examination of a greater number of cells at different stages of infection than was possible in this work. The structures may represent damaged mitochondria, endoplasmic reticulum or less likely golgi apparatus all of which may be altered in appearance during a variety of degenerative and infectious processes (Trump and Bulger, 1967, 1968; Trump and Ginn, 1968; Nakayama et al, 1969; Roy, 1967).

CSM produced cytopathic changes in PK15 cells at infecting dose of 8.26 $\log_{10}$ organisms per ml with higher concentrations producing earlier and more severe cytopathic changes. An increased inoculum is more likely to infect more cells than fewer organisms. At CSM concentration of 11.26 $\log_{10}$ organisms per ml the bacteria/cell ratio is $\geqslant 10^6$:1 while at CSM concentration of 5.26 $\log_{10}$ organisms per ml the ratio is $\leqslant$ 10:1. The infecting dose of 5.26 $\log_{10}$ organisms per ml did not produce any cytopathic changes (Table 3a). At this concentration the bacteria/cell ratio may approach 1:1. The consequence may be that many cells are not infected by bacteria and immunoflourescence and cultural recovery of CSM suggests this view. There is the possibility that in an infecting dose only some organisms attach to cells and of those that are internalised some are destroyed by the cells protective mechanisms. At CSM concentration of 11.26 $\log_{10}$ organisms per ml the bacteria/cell ratio is 10^6:1 and this large bacteria/cell ratio ensures that each cell is exposed to infection. It is possible that the mere

weight of infection itself may affect the survival of CSM through alteration of the gaseous environment or other means.

The increase in PK15 cell concentration affected the time at which cytopathic changes were produced by CSM and also their severity (Table 4). At concentrations below 10^5 cells per ml CSM produced cytopathic changes in the cells more rapidly than at higher cell concentrations. At seeding concentrations of 10^5 cells per ml about 5% of the cell population in monolayer were refractory to infection by CSM. It appears that an increase in the cell concentration increases the number of refractory cells in the cell population and this affects the production of cytopathic changes by CSM. The cell/bacteria ratio is $1:10^7$ at cell concentration of 10^3 cells per ml, $1:10^5$ at 10^5 cells per ml, 1:100 at 10^8 cells per ml and 10:1 at 10^{11} cells per ml. The cell/bacteria ratio at high cell concentrations are similar to the bacteria/cell ratio at high CSM concentrations (Experiment 3.3). An increase in number of cells increases the cell/bacteria ratio which will reduce the number of bacteria that infect cells and hence affect the production of cytopathic effects. At seeding concentration of 10^{11} cells per ml cell monolayers reach confluence after 24 hours. Thereafter the cells grow in multilayers and there is a possibility that some of the permissive cells were mechanically protected from infection.

It is not known whether the progressive intracellular infection of PK15 cells with CSM occurs because some bacteria are different or because the increase in the numbers of bacteria block the defence mechanisms of the cell.

For comparative studies therefore, CSM inoculum and cell density should be reasonably constant. The system developed in this work

appeared a useful base from which to examine the pathogenicity of CSM for cell culture. The system developed in this present work seems to provide a satisfactory base for further studies on the pathogenicity of CSM in cell cultures.

CHAPTER 4.

Chapter 4

Attachment of *Campylobacter sputorum* subspecies *mucosalis* to cells *in vitro*

b) Materials and Methods

c) Results

d) Comments

4.4.4 The effect of the stage of cell growth on the attachment of CSM to cells

a) Introduction

b) Materials and Methods

c) Results

d) Comments

4.4.5 Recovery of CSM from the supernatant fluids of PK15 cell cultures infected at different stages of growth

a) Introduction

b) Materials and Methods

c) Results

d) Comments

4.4.6 The effect of hydroxyurea on PK15 cells and on the attachment of CSM to treated cells

a) Introduction

b) Materials and Methods

c) Results

d) Comments

4.4.7 Synchronisation of PK15 cells by serum-starvation and exposure to hydroxyurea and evaluation of synchrony

a) Introduction

b) Materials and Methods

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d) Comments

4.4.8 Semi quantitative assessment of the attachment of CSM to synchronised PK15 cells

a) Introduction

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4.5 Determination of PK15 cell surface receptors for CSM adhesins

4.5.1 Introduction

4.5.2 The effect of lectins on the attachment of CSM to PK15 cells

a) Introduction

b) Materials and Methods

c) Results

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4.5.3 The effects of CSM-antiserum, enzymes, salts of heavy metals and hydroxyurea on attachment of CSM to PK15 cells

a) Introduction

b) Materials and Methods

c) Results

d) Comments

4.6 Comparison of the attachment of CSM and E. coli to pig brush borders

4.6.1 Introduction

4.6.2 Materials and Methods

4.6.3 Results

4.6.4 Comments

4.7 Discussion

Chapter 4

Attachment of *Campylobacter sputorum* subspecies *mucosalis* to cells *in vitro*

4.1 General Introduction

Selective adherence of pathogenic bacteria to the surfaces of epithelial cells of mucous membranes appears to be the first stage in the initiation of infection in the gastrointestinal tract (McNeish, Turner, Fleming and Evans, 1975), genitourinary tract (Ward and Watt, 1972, Stamey *et al*, 1978) and respiratory tract (Ellen and Gibbons, 1974, La Force *et al*, 1976, Powell *et al*, 1976, Sugarman and Donta, 1979). For many pathogenic organisms adherence is of paramount importance since they have to compete with commensal microorganisms for successful colonisation of the host epithelial cell surfaces. Specific adhesive mechanisms have been found to play an important role in the attachment of various members of the Enterobacteriaceae, Neisseriaceae, Streptococcaceae and Mycoplasmas to different epithelial tissues (Chapter 1).

To colonise mucosal surfaces the organisms must first bind to the epithelial cells of these tissues or they are eliminated by a variety of host defence mechanisms. Some bacteria, such as *Shigella* spp (Takeuchi *et al*, 1968) attach to mucosal epithelial cells, penetrate into the epithelial cells, multiply and kill the cells thus indicating disease by eroding and ulcerating the mucosal epithelium. Other bacteria attach to mucosal epithelial cells but do not penetrate. These organisms multiply on the surface and elaborate exotoxins that pass into the epithelium, an example of this type of relation is *Vibrio cholerae*

(Freter, 1969; Finkelstein, 1969).

In recent years, the phenomenon of bacterial attachment to cell surfaces has attracted attention mainly because its specificity provides a basis for explaining the susceptibility of the host at the cellular level. Bacterial adhesion studied *in vitro* should ideally involve the same specific mechanisms of mucosal association that take place *in vivo*. Campylobacter-like profiles, possibly CSM, have been regularly demonstrated in the cytoplasm of adenomatous intestinal epithelium (Rowland and Lawson, 1974; Roberts, 1978) but the stage of cell invasion has never been adequately observed or described. It would appear reasonable however to suggest that cell infection involves initially attachment to and thereafter penetration of intestinal epithelial cells.

CSM attaches avidly to certain pig cell lines, the work in this chapter describes an examination of this phenomenon and a comparison of attachment to these and intestinal cells. Initial experiments were undertaken to examine the mechanisms of adherence of CSM mainly to PK15 cells.

4.2 Attachment of CSM to the surfaces of PK15, MDBK and Int 407 cells

4.2.1 Introduction

Several workers have shown that a variety of pathogenic bacteria are able to attach to cell cultures, and the relevant literature has been reviewed in Chapter 1.

The following experiment was designed to compare the attachment of six strains of CSM of different serotypes (Table 1) to PK15 cells.

One strain was later used to assess the attachment of CSM to preformed monolayers of PK15, MDBK and Int 407 cells.

4.2.2 Materials and Methods

i) Preparation of bacterial inocula

The strains of CSM used in this experiment were:- 253/72, 302/72, 140/76-220, 722/75, 982/76 and 512/77. Procedures for routine growth, maintenance of these organisms on bacteriological media, and preparation of inocula have been described previously (2.5.1, 2.5.3).

ii) Sources of cell cultures

The cell lines used were obtained by normal purchase as follows:- PK15 cells (Gibco (Biocult) Europe Ltd., Paisley, Scotland), MDBK cells (Flow Laboratories, Irvine, Ayrshire, Scotland) and Int 407 cells (Flow Laboratories, Irvine, Ayrshire, Scotland).

iii) Infection of cell cultures

In the first experiment, 24 hour-old preformed coverslip cultures of PK15 cells were overlaid with 1 ml of a suspension of each CSM strain and incubated at 37°C. These and uninfected control cultures were examined at 20 minute intervals for the first hour, thereafter at two-hourly intervals over 12 hours and at 24 and 48 hours post-infection. After thorough rinsing in several changes of warm PBS to remove unattached bacteria, the monolayers were processed for scanning, transmission electron microscopy, immunofluorescence and Giemsa staining by the methods described in Chapter 2.

In the second experiment, a semi-quantitative assessment of the attachment of CSM strain 253/72 to PK15, MDBK and Int 407 cells was

attempted. Giemsa stained coverslip monolayers were prepared at two-hourly intervals for the first 12 hours and at 24 and 48 hours post-infection. About 100 cells on each coverslip were examined at random for the presence of attached bacteria. Because bacterial attachment may vary from cell to cell at a particular time, cultures were arranged into 4 groups on the basis of the number of attached bacteria, namely:- 0, 1-10, 11-20 and 20 organisms per cell. The largest group of cells showing a particular pattern of bacterial attachment at a given time was taken as a representative picture of the adhesive process.

4.2.3 Results

Table 5a: Attachment of different strains of CSM to PK15 cells

Time after infection in minutes (m) or hours (h)	CSM Strain 253/72	302/72	140/76-220	772/75	982/76	512/77
0m	-[a]	-	-	-	-	-
20m	++	++	++	++	++	++
40m	+++	+++	+++	+++	+++	+++
60m	+++	+++	+++	+++	+++	+++
2h	+++	+++	+++	+++	+++	+++
4h	+++	+++	+++	+++	+++	+++
6h	++	++	++	++	++	++
8h	++	++	++	++	++	++
10h	+	+	+	+	+	+
12h	+	+	+	+	+	+
24h	-	-	-	-	-	-
48h	-	-	-	-	-	-

a Predominant cell/bacteria attachment type:

+ = Most of the cells showing less than 10 attached bacteria per cell

++ = Most of the cells showing 11 to 20 attached bacteria per cell.

+++ = Most of the cells showing more than 20 attached bacteria per cell.

- = No bacteria attached to cell surface.

Table 5b: Semi-quantitative assessment of the attachment of CSM strain 253/72 to cell cultures

Time after infection in hours	Degree of attachment to 3 species of cell cultures					
	PK15		MDBK		Int 407	
2	+++[a]	(95)	+	(11)	+	(5)
4	+++	(90)	+	(29)	+	(10)
6	++	(79)	++	(40)	+	(12)
8	++	(50)	++	(52)	++	(22)
10	+	(29)	+	(21)	++	(29)
12	+	(19)	+	(8)	++	(50)
24	-	(0)	-	(0)	+++	(98)
48	-	(0)	-	(0)	+++	(98)

a Predominant cell/bacteria attachment type:

\+ = Most of the cells showing less than 10 attached bacteria per cell

++ = Most of the cells showing 11 to 20 attached bacteria per cell.

+++ = Most of the cells showingmore than 20attached bacteria per cell.

\- = No bacteria attached to cell surface.

( ) Numbers in brackets indicating the percentage of cells showing bacteria attached to the cell surface.

i) Attachment of different strains of CSM to PK15 cells

Giemsa-stained preparations of PK15 cells obtained after exposure to each of the six CSM strains showed a similar pattern of bacterial attachment throughout the period of the experiment (Table 5a). After 20 minutes of infection about 40% of the cells had organisms (about 11-20 per cell) attached to the cell surface. The attachment of all strains used reached a maximum around 2-4 hours post infection. After this time there was a continuous decrease in the number of bacteria on the cell surface until 24 hours post infection when no organisms were seen.

ii) Semi-quantitative assessment of the attachment of CSM strain 253/72 to cell cultures

Giemsa staining and scanning electron micrographs of PK15, MDBK and Int 407 cells infected with CSM showed that after 2 hours of infection bacteria were adherent to the surfaces of about 95, 11 and 5% respectively, of the cell populations (Table 5b, Fig. 11). At this stage PK15 cells had relatively large numbers of bacteria attached to the surfaces while MDBK and Int 407 cells had few organisms (less than 10 per cell). CSM attachment reached a maximum around 2-4 hours post infection for PK15 cells, 6-8 hours for MDBK cells and 24-48 hours for Int 407 cells. During this "active" phase of attachment large numbers of bacteria were present over the whole cell surface of Int 407 cells, whereas in PK15 and MDBK cells adhesion was mainly restricted to the cell periphery (Figs. 12a-c, 13, 14a,b). After the "active" phase of attachment in PK15 and MDBK cultures there was a progressive decrease in the number of adherent bacteria. In Int 407 cells however, attachment increased with time

Fig. 11. Scanning electron micrograph of PK15 cells 2 hours after infection with CSM. Numerous bacteria attached to the cells but one cell (arrowed) did not show organisms on the cell surface.

(x 4333)

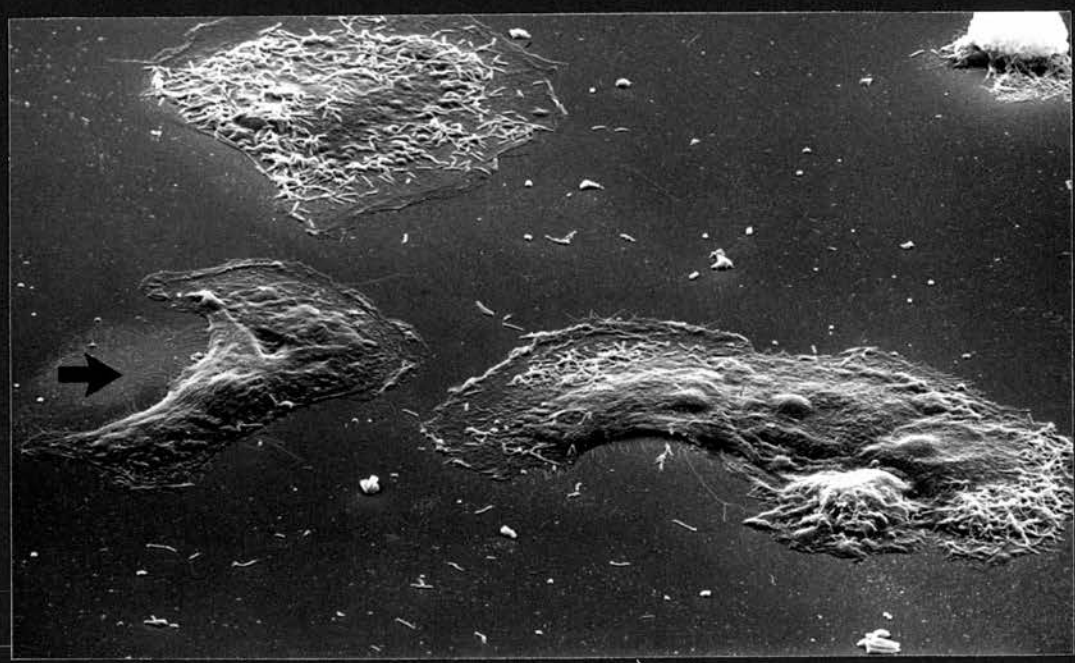
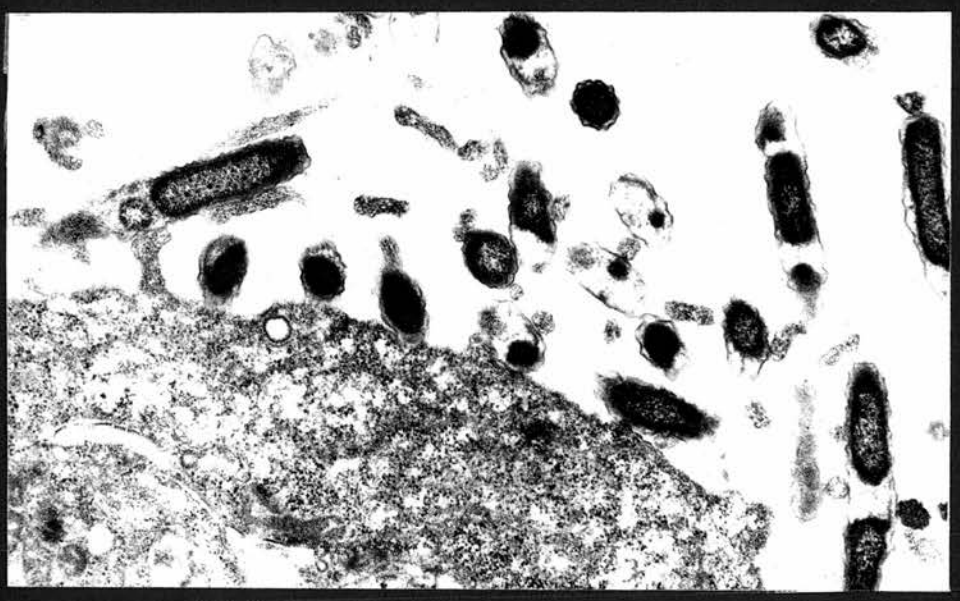


Fig. 12a. PK15 cells 2 hours after infection with CSM. Large masses of brightly stained vibrioid forms attached to the periphery of the cells.
Acetone fixation, immunofluorescence staining with anti-CSM serum and sheep anti-rabbit (FITC) conjugate.
(x 284)

Fig. 12b. Transmission electron micrograph of PK15 cells 2 hours after infection with CSM showing bacteria attached to the cell surface. Bacteria contact does not appear to involve a specific area of the bacterial envelope.
(x 12500)

Fig. 12c. Scanning electron micrograph of PK15 cells 2 hours after infection with CSM. Bacteria are attached to the cell surface.
(x 10800)



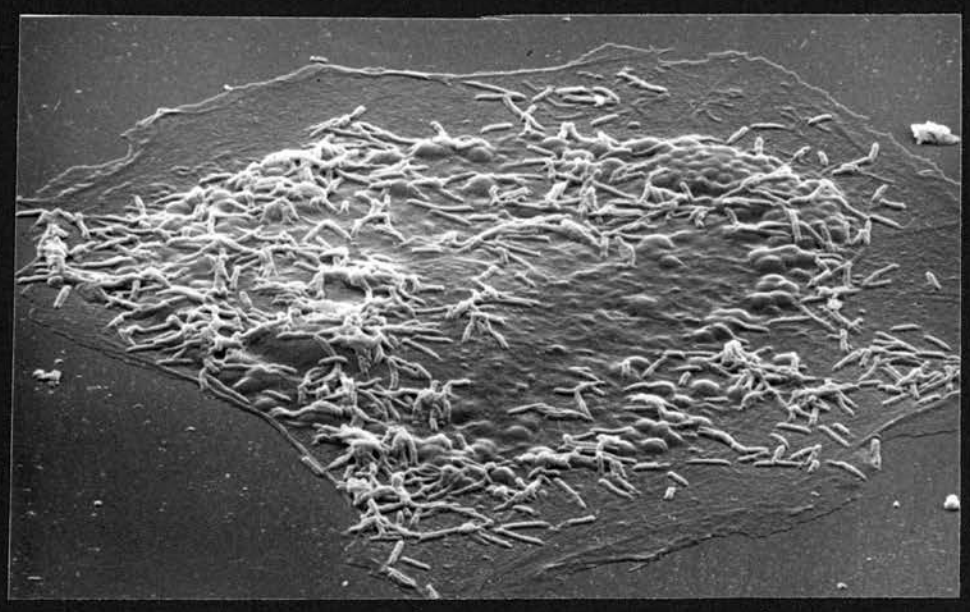


Fig. 13. Scanning electron micrograph of MDBK cells 6 hours after infection with CSM. Only a few organisms are attached to the cell surface and bacteria are not concentrated at the cell periphery.

(x 6975)

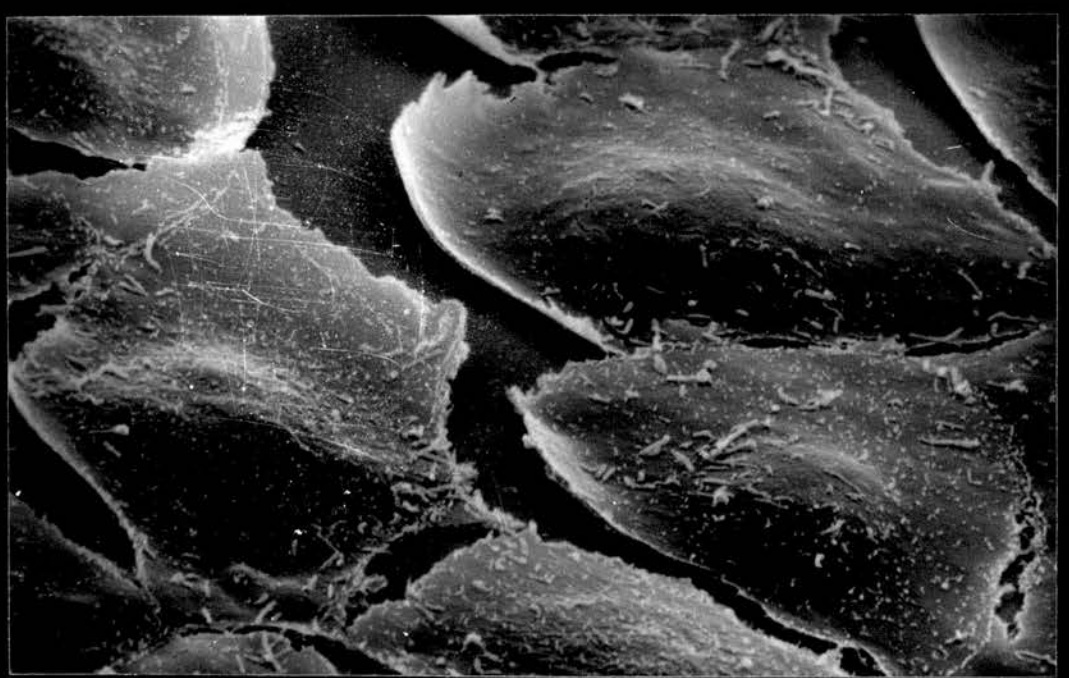
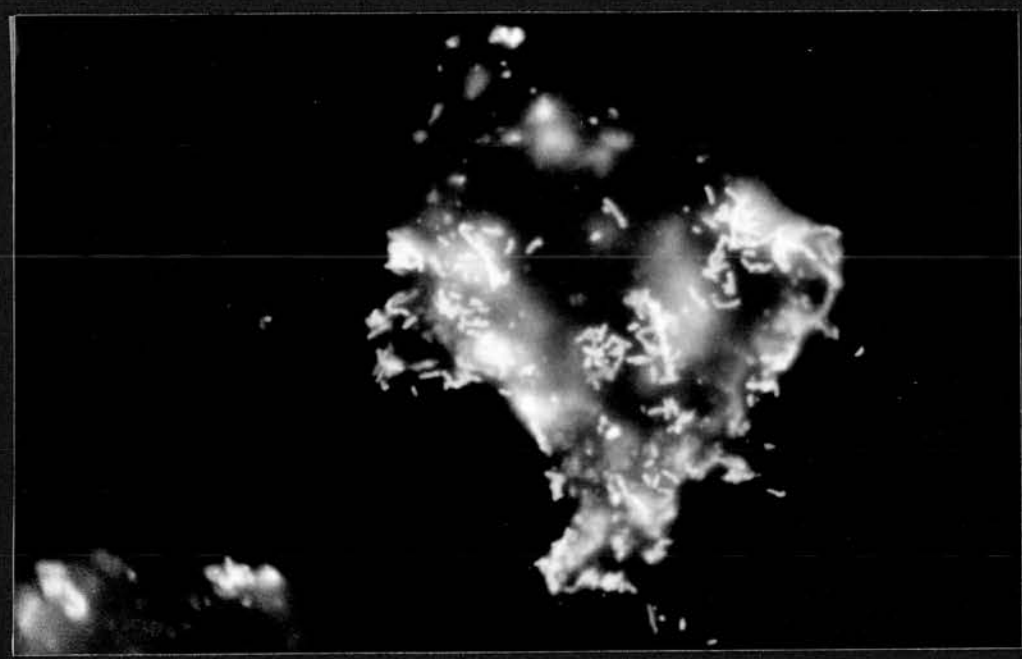
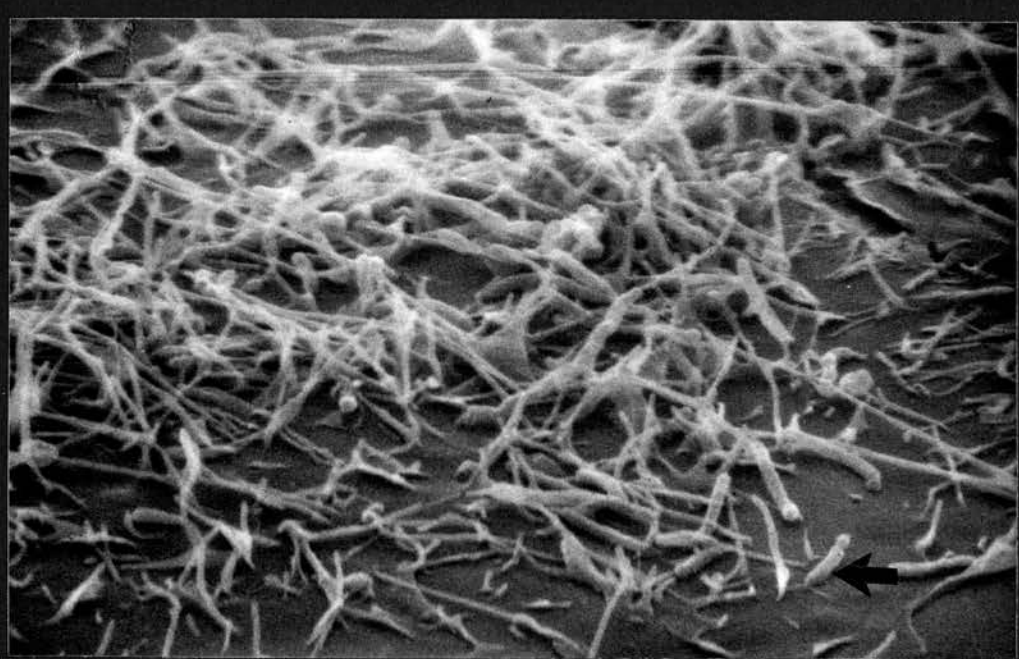


Fig. 14 a. Int 407 cells 24 hours after infection with CSM. Vibrioid forms are attached all over the cell surface.
Acetone fixation, immunofluorescence staining with anti-CSM serum and sheep anti-rabbit (FITC) conjugate.
(x 284)

Fig. 14b. Scanning electron micrograph of Int 407 cells 48 hours after infection with CSM. Numerous attached organisms (arrowed) amongst cellular processes.
(x 23400)





and at 12 hours post-infection some 50% of the cell population showed 11-20 adherent organisms.

CSM was not seen on cell surfaces of PK15 or MDBK cells after 24 and 48 hours respectively while at this time 90-98% of Int 407 cells showed more than 20 organisms per cell.

4.2.4 Comments

i) These observations clearly indicate that different strains of CSM attach to PK15 cells in the same pattern and with similar degrees of intensity. Although, certain of these strains of CSM have very different surface antigens from the serotype A strains (982/76 serotype B, and 512/77 serotype C), this does not seem to vary the pattern or intensity of attachment to PK15 cells. This suggests multiple receptors, common receptors or that the preparative method for agglutinating antigens destroys a common bacterial surface antigen.

ii) Bacterial attachment to preformed monolayers during the peak period was greater in PK15 than MDBK cells. Organisms attached all over the surface of Int 407 cells, while the bacteria adhered preferentially to the periphery of PK15 and MDBK cells. The pattern of attachment obtained on Int 407 cells infected with viable CSM has also been described by Rajasekhar (1981) on PK15 cells infected with air-inactivated CSM and, more recently, by Scaletsky *et al* (1984) in Hela cells infected with *E. coli*. In the latter case the pattern of attachment showed a different distribution of organisms according to the strain of *E. coli* examined. However, since viably-different CSM attached in a similar manner to cultured cells from two different species this might suggest that the receptors for air-inactivated CSM

on PK15 cells are similar to those for viable CSM on Int 407 cells although a number of other explanations are equally possible.

The demonstration of organisms on the cell surface of infected Int 407 cells up to 48 hours after infection was different from the pattern observed in PK15 and MDBK cultures where no organisms were seen on cell surfaces. This suggests that removal of bacterial from the cell surface is a specific process that takes place with CSM in PK15 and MDBK cell lines but not Int 407 cells. That Int 407 cell lines are capable of taking up bacteria has been shown by Newell and Pearson (1981) with C. jejuni.

4.3 The effect of the age of CSM culture on bacterial attachment to PK15 cells

4.3.1 Introduction

Previous infections of cells in culture employed 24 hour-old cultures of CSM. Because the age of CSM culture was considered to be a factor that may affect CSM attachment to cells the following experiment was performed to compare the attachment of cultures of CSM of different ages to PK15 cells.

4.3.2 Materials and Methods

Five CBA slopes were inoculated with CSM and incubated at 37°C microaerophilically. Every day the surface growth of one slope was washed off with warm PBS and the number of organisms in the suspension determined by surface viable counts. One ml of each suspension was used to infect separate 24 hour-old PK15 cells grown on coverslips and

incubated at 37°C. Coverslips were removed after 1 hour, washed with warm PBS, fixed in methanol and stained by Giemsa's method. Assessment of motility of the bacterial culture was carried out by placing a drop of the culture suspension in a cavity slide covering it with a coverslip and viewing it under the light microscope.

4.3.3 Results

On each of the first 3 days of incubation the motility of CSM was marked and of similar intensity but, by the 4th and 5th days, cultures showed only a few motile organisms. The intensity of attachment decreased as the cultures became older until with 5 day-old culture no attachment was seen (Table 6).

Bacterial forms changed from curved rods in the first day to a population which was made up of short rod and cocco-bacillary forms by the 3rd and subsequent days. In contrast to the organisms in 24 hour-old cultures these latter forms stained poorly with conventional bacterial strains.

It is emphasised that the viable bacterial counts of CSM of all the inocula were in the range that normally produced maximum attachment to PK15 cells.

Table 6: Effect of age of CSM cultures on bacterial attachment to PK15 cells

Age of CSM culture in days	Motility of bacteria	Intensity of attachment	Bacterial form	Inoculum expressed as $\log_{10}$ organisms per ml.
1	3[a]	+++[b]	R	10.57
2	3	+++	R	10.74
3	3	++	R/C	11.40
4	2	+	R/C	11.54
5	1	-	C	10.68

a Motility of bacteria: 1 through 2 to 3 = few to many motile bacteria

b Bacteria attached to surface of cell: - = no bacteria, + to +++ = increasing numbers of bacteria

R = Curved rods and vibrio-like forms

C = Short rods and coccobacillary forms

4.3.4 Comments

Previous workers (Rajasekhar, 1981) and Newell and Pearson (1981) have suggested that bacterial motility is involved in the attachment of campylobacters to cells in culture and the present results also indicate that attachment of CSM to PK15 cells is influenced by the motility and/or age of the bacterial inoculum.

It would also appear that the change of bacteria from curved rods to poorly stained cocco-bacillary forms with age is associated with a loss of motility and this in time may affect bacterial attachment.

Thus the age of the CSM cultures affects bacterial adhesion to PK15 cells and it is important that young cultures are used in testing adherence to tissue culture cells. For this reason the standard procedures hereafter always employed 24 hour-old cultures of CSM.

4.4 The refractory nature of some of the cells in monolayer cultures to CSM adherence

4.4.1 Introduction

It has been shown in preliminary infection experiments with CSM that the bacteria do not adhere to all the cells in a population. This feature could be the result of a variety of factors such as (i) the number of organisms in the inoculum (ii) cell to cell contact in the monolayer preventing bacterial access to the surface, or (iii) the stage of development of some cells when exposed to infection with CSM.

The following experiments investigated the possible role

played by these variables in these "non-receptive" cells, and to find out if CSM adheres to all the cells in a population.

4.4.2 The effect of infecting dose on the attachment of CSM to PK15 cells

It has been shown in Chapter 3 that the weight of inoculum has a marked effect on the cytopathic changes in PK15 cells. At lower levels of infection no cytopathic effects were detected and the infection did not persist. This observation could be explained by small inocula failing to attach to the cells or alternatively that whilst bacterial attachment is normal, successful cell infection requires a number of bacteria to adhere to each cell surface.

The following experiment was performed to examine the effect of inoculum on the attachment of CSM to "receptive" and "non-receptive" cells in monolayer. PK15 cells were infected with varying concentrations of CSM and the percentage of cells showing attached bacteria at the peak period of attachment noted for each CSM concentration.

a) Materials and Methods

Three dilutions of CSM were prepared as in experiment 3.3.2 and the number of organisms in each dilution determined by surface viable count. One ml of each dilution was separately used to infect 24 hour-old PK15 cells grown on coverslips and incubated at 37°C. Coverslips were removed hourly for 6 hours, fixed in methanol and stained by Giemsa's method.

b) Results

Table 7: Attachment of CSM to PK15 cells: effect of inoculum size

Time after infection in hours	Bacterial inocula expressed as $\log_{10}$ organisms per ml					
	10.63		7.63		4.63	
1	+++[a]	(94)	++	(90)	+	(71)
2	+++	(95)	++	(89)	+	(70)
3	+++	(95)	++	(89)	+	(70)
4	+++	(95)	++	(89)	+	(70)
5	++	(95)	+	(89)	+	(69)
6	++	(95)	+	(88)	+	(69)

a Predominant cell/bacteria attachment type:

\+ = Most of the cells showing less than 10 attached bacteria per cell.

++ = Most of the cells showing 11 to 20 attached bacteria per cell.

+++ = Most of the cells showing 20 or more attached bacteria per cell.

( ) = Numbers in brackets indicating the percentage of cells showing bacteria attached to cell surface.

At CSM concentration of 10.63 $\log_{10}$ organisms per ml about 95% of the cells showed bacterial attachment. At concentrations of 7.63 $\log_{10}$ and 4.63 $\log_{10}$ organisms per ml, there were marked reductions in both the number of cells showing attached bacteria and the number of organisms that were attached to the cell surfaces (Table 7). Very few organisms (about 2-3 per cell) attached to PK15 cells at CSM concentrations of 4.63 $\log_{10}$ organisms per ml.

c) Comments

The weight of inoculum of CSM used to infect PK15 cells has an effect on both the number of organisms that attach to the cells and the number of cells that show bacterial attachment. At CSM concentration of 10.63 $\log_{10}$ organisms per ml the bacteria to cell ratio is high, and this is evident in the intensity of attachment and number of cells showing adherent bacteria (Table 7). At high concentrations of bacteria the absence of suitable bacteria to demonstrate "receptive" cells is unlikely. The apparent increase in "non-receptive" cells at lower inocula could be due to a combination of "non-receptive" cells and a lower availability of suitable bacteria capable of adhering. One should not ignore the possibility that this increase in "non-receptive" cells may be due to the presence of some cells with very small numbers of adherent bacteria which may not be accurately recorded. A more profound effect than could be accounted for merely by the numbers of bacteria should not be ignored as Rajasekhar (1981) showed that cytopathic effects could be induced in otherwise refractory cell lines by repeated infection with CSM.

4.4.3 The effect of cell to cell contact on the attachment of CSM to PK15 cells

a) Introduction

PK15 cells grow to a confluent monolayer in coverslips after 24 hours at cell inocula of 1 x 10^5 cells per ml. At this time the cells are sometimes in contact with neighbouring cells in the monolayer and these contact points in contrast to the free cell margin do not show bacterial attachment when infected with CSM.

Cells in suspension are not in contact with one another, so the effect of cell to cell contact on the number of cells to which CSM adhere can be studied by infecting cells in suspension with the bacteria.

b) Materials and Methods

PK15 cells were grown in medical flats for 24 hours, trypsinised and resuspended at 1 x 10^5 cells per ml in MEM with Earle's salt, L-glutamine and 0.3% methyl acetate (for suspending cells). Fifty ml of the suspended cells were continuously agitated in a stirrer flask at 37°C for the length of the experiment. An equal volume of 24 hour-old culture of CSM in warm MEM was added to the cells after 24 hours in the suspension flask. At hourly intervals for six hours, 5 ml of the infected cell suspension was withdrawn and centrifuged three times in warm PBS at 200 x *g* for 1 minute. The supernatant fluid was discarded, the pellet resuspended in a few drops of warm PBS and spread on a glass slide. The preparation was air-dried, fixed in methanol and Giemsa-stained. The number of cells out of 100 showing adherent bacteria were counted under the light microscope.

c) Results

Table 8: Attachment of CSM to suspended PK15 cells and percentage of cells showing adherent bacteria

Time after infection in hours	Intensity of bacterial attachment	Percentage of cells showing bacterial attachment
1	+++	98
2	+++	98
3	+++	98
4	+++	98
5	++	98
6	++	97

++ = Most of the cells with 11 to 20 adherent bacteria per cell

+++ = Most of the cells withmore than 20adherent bacteria per cell.

Cells that showed CSM attachment had bacteria all around their periphery and 97-98% of cells in suspension showed organisms attached to their surface (Table 8).

d) Comments

CSM attaches to about 95% of PK15 cells in preformed monolayers. In cell suspension, CSM attaches to about 98% of the cells. This is an apparent increase from the number of cells that show adherent bacteria in monolayer.

Too much emphasis should not perhaps be placed on these differences as suspension culture itself may possibly modify receptor sites. The experiment does however indicate that even where cell contact is minimised there appears to be a relatively constant proportion of the cells to which CSM does not attach. The difference in the number of "receptive" cells may be due to an abolition of cell contact but clearly the cells in suspension culture may differ in other ways from cells in monolayer.

4.4.4 The effect of the stage of cell growth on the attachment of CSM to cells

a) Introduction

The infecting dose of CSM and possibly cell to cell contact have been shown to play some parts in the non-attachment of CSM to certain cells in a culture. However, even a large inoculum and the use of

suspended cells did not achieve attachment of CSM to all the cells in the culture.

In this next experiment, PK15 cells at different stages of growth were infected with CSM in an examination of the effect of the stage of maturity of the cell on attachment.

b) Materials and Methods

A confluent monolayer of PK15 cells grown for 7 days in a 4 oz medical flat was detached from the glass with a sterile rubber policeman and not with STV. The contents of the medical flat were centrifuged at 200 x g for 1 minute, the supernatant fluid discarded the pellet resuspended in growth medium and seeded into test tubes containing coverslips.

Cell monolayers were infected with CSM at 4 hourly intervals from the initial cell seeding for the first 24 hours and thereafter daily for 3 days. Infection of coverslips was carried out as previously described in Chapter 2. The infected monolayer cultures were removed after 1 hour of incubation at 37°C, washed in warm PBS, fixed in methanol and stained by Giemsa's method.

c) Results

Table 9a: Effect of the stage of cell growth on the attachment of CSM

Age of cell culture at time of infection in hours	Intensity of attachment and percentage of cells showing bacteria		CSM inoculum expressed as $\log_{10}$ organisms per ml
4	-[a]	(0)	10.60
8	-	(0)	10.35
12	+	(63)	11.30
16	++	(88)	11.18
20	+++	(92)	10.72
24	+++	(95)	10.65
48	++	(90)	11.18
72	+	(62)	11.30

a Predominant cell/bacteria attachment type:

\+ = Most of the cells showing less than 10 attached bacteria per cell

++ = Most of the cells showing 11 to 20 attached bacteria per cell

+++ = Most of the cells showingmore than 20attached bacteria per cell

\- = No bacteria attached to cell surface

( ) = Numbers in brackets indicating the percentage of cells showing bacteria attached to cell surface.

During the first 8 hours of cell growth, CSM did not adhere to PK15 cells. The number of organisms that attached to cell surfaces was greatest between 20 and 24 hours of cell growth. The inoculum dose used on each occasion was in the range that yields maximum attachment of CSM to PK15 cells in monolayer (4.4.2). At 48 or 72 hours of PK15 growth there was a decrease in the cells demonstrating attached bacteria and the number of adherent organisms.

d) Comments

PK15 cells were refractory to the attachment of CSM during the first 8 hours of cell-growth (Table 9a). In an earlier trial experiment where cells had been detached from glass with trypsin, CSM did not attach to PK15 cells during a similar period of growth. An explanation for the inability of CSM to attach to these cells might have been the destruction of CSM receptor sites on the cells by trypsin. The subsequent experiment described here was performed on cells that were mechanically detached from the glass.

After 24 hours incubation CSM attached to 95-98% of cells in monolayer or in suspension. Attachment of bacteria to cells in suspension indicates that the inability to attach in the first hours of cell growth is not related to adherence of the cells to glass and it appears that the stage in cell growth at the time of infection with CSM affects the attachment to PK15 cells.

4.4.5 Recovery of CSM from the supernatant fluids of PK15 cell cultures infected at different stages of growth

a) Introduction

It has been shown in 4.4.4 above that CSM did not attach to PK15 cells in the first 8 hours of growth. It is known that the bacteria are rapidly killed in growth medium with or without refractory cells under aerobic conditions. This system can be used to confirm the refractory nature of cells to bacterial infection by exposing cells to the organisms and assessing whether bacteria can be recovered from the supernatant fluid.

With this in mind PK15 cells were grown in coverslips and infected with CSM during the first 8 hours of growth. Daily viable counts of the supernatant fluids were performed to ascertain whether there was persistence of the organism.

b) Materials and Methods

A similar experiment to that described in 4.4.4 (b) was undertaken. The cells were infected with CSM at 2, 4, 8, 12 and 24 hours of growth. Infection of coverslips was carried out as previously described in Chapter 2. The recovery of organisms from the supernatant fluid of infected cells was determined daily for 3 days by the surface viable count method as previously described in Chapter 2. The recovery of organisms from infected 24 hour-old PK15 cells was used as control.

c) Results

Table 9b: The recovery of CSM from supernatant fluid of PK15 cells infected with bacteria at different stages of growth

Time after infection in days	Cell culture age at the time of infection in hours				
	Control 24	4	8	12	24
1	7.40	NR	NR	7.18[a]	6.88
2	7.70	NR	NR	7.30	7.18
3	7.18	NR	NR	6.98	6.74

a = Bacterial count expressed as $\log_{10}$ per ml (corrected to two decimal places).

NR = No organisms recovered

CSM was not recovered from the supernatant fluids of PK15 cells infected during the first 8 hours of growth at any time in the 3 days of the experiment. Bacteria were recovered from control cells and from cells infected at 12 or 24 hours.

d) Comments

The inability to recover CSM from the supernatant of PK15 cells infected during the first 8 hours of cell growth further confirms the refractory nature of these cells to attachment of the bacteria at this stage. If these cells had not been refractory during this period, bacteria should have been recovered from the supernatant fluids as was the case for cells infected after 12 or 24 hours of growth.

4.4.6 The effect of hydroxyurea on PK15 cells and on the attachment of CSM to treated cells

a) Introduction

The generation time of cells in a population differ as a result of the sum of small differences in the times taken for the large numbers of steps required for a cell to progress from one division to the next. As a consequence, in any given population cells will be found at different stages in the cycle of cell division. In the previous experiments cells appeared refractory to attachment in the hours following seeding at a time when the cells are dividing actively.

Attempts were made in this next experiment to synchronise cells

with a growth inhibitor, hydroxyurea. Hydroxyurea inhibits DNA synthesis by its action on ribonucleotide reductase and this action is readily reversed by replacing the medium with drug-free medium. In subsequent experiments the synchronised cells were infected with CSM to assess the attachment to PK15 cells at different stages in the cell growth cycle.

A number of growth inhibitors used for synchronisation of cells are known to be toxic to some types of cell lines. With this in mind, the present experiment first examined the effect of and the toxicity of hydroxyurea to PK15 cells and, secondly, the attachment of CSM to PK15 cells previously modified by hydroxyurea.

b) Materials and Methods

i) Effect of hydroxyurea on PK15 cells

PK15 cells were grown in MEM with 1% calf serum in 200 ml medical flats for 3 days. The medium was removed, the monolayers washed thoroughly in MEM and the cells refed with the normal growth medium containing 10% calf serum. Six hours later, concentrated hydroxyurea stock solution was added to each medical flat to reach final concentrations of 1, 2 or 3 mM hydroxyurea. These cells were exposed to hydroxyurea in growth medium for 14 hours, then the medium was removed, the monolayer washed with warm medium and refed with growth medium without hydroxyurea. Cells were detached from the glass by trypsinisation, centrifuged and resuspended in 10 ml warm growth medium, then dispensed in 1 ml amounts into test tubes with coverslips. Cells were incubated at 37°C for 4 hours after which they had attached to the coverslips. The coverslip monolayers were washed in warm PBS, fixed in

methanol and stained by Giemsa's method. Cells not exposed to hydroxyurea but otherwise treated in the same manner and grown on coverslips for 4 hours were also stained and used as controls.

ii) Effect of hydroxyurea on attachment of CSM to PK15 cells

Cells exposed to hydroxyurea and grown on coverslips for 4 hours were infected with 1 ml of a 24 hour-old culture of CSM suspended in warm MEM. Infected cells were incubated at 37°C for 1 hour, the coverslips were rinsed in warm PBS, fixed in methanol and stained by Giemsa's method. Cells not exposed to hydroxyurea and grown on coverslips for 24 hours prior to infection with 1 ml of CSM suspension were used as controls.

c) Results

i) Effect of hydroxyurea on PK15 cells

Giemsa-stained preparations of cells exposed to 1 mM hydroxyurea were not different from unexposed control cells. There were similar numbers of cells (about 8%) that were in different stages of mitotic division.

The cytoplasm of cells exposed to 2 or 3 mM hydroxyurea showed very many cytotoxic vacuoles. The majority of the cells were in stages of division.

ii) Effect of hydroxyurea on attachment of CSM to PK15 cells

Although 2 and 3 mM hydroxyurea were toxic to PK15 cells,

neither concentration hindered the attachment of CSM to those cells. The attachment of organisms to these cells was more dense and tight than that of CSM to unexposed control cells and followed the same pattern being more intense at the cell periphery than elsewhere.

d) Comments

It is not known whether the apparently more avid attachment seen in cells previously exposed to hydroxyurea was a result of "synchronisation" of cells or modification of cell surface receptors by this growth inhibitor. Attempts were made in later experiments to examine this effect.

4.4.7 Synchronisation of PK15 cells by serum starvation and exposure to hydroxyurea and evaluation of synchrony

a) Introduction

Most tissue culture cells especially those of virally transformed cell lines are asynchronously distributed throughout the cell cycle even when growth is markedly slowed down by nutritional deficiencies (Bartholomew et al, 1976). Since metabolic activities are different in the phases of the cell cycle, synchronised populations of cells are useful and necessary for the study of cell cycle related events.

Burk (1970) showed that Syrian hamster cells (BHK 21/C13) failed to grow when transferred to a medium containing 0.25% serum and could be maintained in this medium in a quiescent state for 8 days or more. On the addition of normal levels of serum no DNA synthesis recurred for 9 hours and thereafter mitotic peaks were observed at about 23 and 33 hours.

Pre-treatment of serum-starved cells with either hydroxyurea or cytosine arabinoside before they were stimulated to proliferate by the addition of medium with 10% serum increased the degree of synchrony during DNA synthesis (Mironescu and Ellem, 1976).

The extent to which PK15 cells were synchronised by serum-starvation and hydroxyurea was evaluated by autoradiographic studies with tritiated thymidine and light microscopy. Thymidine has been shown to be a specific precursor of DNA (Friedkin et al, 1956) and the low mean β-energy released during the decay of tritium (^3H) facilitates excellent resolution on autoradiographs (Taylor et al, 1957). The incorporation of (^3H) thymidine into the host cell nuclei has been consequently employed as an indicator of DNA synthesis. All cells that have been synchronised will when exposed to (^3H) thymidine incorporate it (^3H) into their DNA at the same time and this activity can be detected by autoradiography.

In this experiment serum-starved PK15 cells were exposed to either 1 or 2 or 3 mM hydroxyurea. Then, the concentration of hydroxyurea that synchronised most cells in a population was determined by autoradiographyic studies and this concentration of hydroxyurea used for subsequent cell synchronisation.

b) Materials and Methods

i) Synchronisation of cells by serum-starvation and exposure to hydroxyurea

PK15 cells were grown in MEM with 1% calf serum in 200 ml medical flats for 3 days. The medium was removed, the monolayers washed thoroughly

in MEM and the cells refed with the normal growth medium containing 10% calf serum. Six hours later, concentrated hydroxyurea stock solution was added to each flat to reach required final concentrations. After the cells had been exposed to hydroxyurea* for 14 hours the medium was removed, the monolayers washed and detached from the glass with STV, centrifuged at 200 x g for 1 minute, resuspended in growth medium and reseeded in test tubes with coverslips.

During the entire process, prewarmed (37°C) medium only was added to cells. This precaution was taken to avoid variations in temperature interfering with the synchronisation of the cells.

* Sigma Chemical Company Ltd., Dorset, England.

ii) Evaluation of the degree of synchrony by autoradiography

The hydroxyurea-treated cells (i) were exposed to 10 μCi/ml (6-^{3}H) thymidine in test tubes (Amersham International, White Lion Road, Amersham, Buckinghamshire, England, specific activity 23 Ci/mmol). After 15 minutes of exposure to tritiated thymidine at 37°C, coverslips were washed with warm MEM and waste medium discarded in appropriate bottles and later disposed of in a "radioactive waste" sink. The coverslips were then air-dried, fixed in methanol and coated by dipping them (Joftes and Warren, 1955) in Ilford L4 nuclear emulsion (Polysciences Inc., Paul Valley Industrial Park, Warrington, Philadelphia 18976, U.S.A.).

Coating and development of coverslips were undertaken in a dark-room using a method modified from that of Williams (1977). A fresh batch of nuclear emulsion was removed from a refrigerator and allowed to rise up to dark-room temperature (18°C) at a constant relative humidity of 60-70% over 45-60 minutes. During this period the safelight

was used with a 15-Watt bulb and the unit covered with Kodak Wratten series 2 filter (Kodak Ltd., Box 33, Swallowdale Lane, Hemel Hempstead, Hertfordshire, U.K.) as the sole light source and the room checked for light-tightness. A specially designed perspex container 78 mm long x 55 mm broad and 40 mm deep to hold the emulsion was partially submerged and firmly positioned in the waterbath previously stabilised at 44-45°C. Five gm emulsion gel were weighed out into a 150 ml beaker, 5ml deionised distilled water added and the beaker then transferred to the water bath for 10 minutes. The molten emulsion was then carefully poured into the pre-warmed perspex container. The coverslips were then dipped in the diluted emulsion and withdrawn vertically and slowly (about ten immersions for each coverslip). Coated coverslips were hung by clothes pegs away from the safelight, dried for 45-60 minutes in front of a fan and stored in 8 x 10 cm black plastic slide boxes (A.R. Horwell Ltd., 2 Grangeway, Kilburn Road, London, England)., containing fresh silica gel in velin tissue, and sealed with black "Scotch" elasticated tape No. 33 (3M. U.K. Ltd., 3M House, Wigmore Street, London, England). Coated coverslip monolayers of cells either exposed or unexposed to hydroxyurea were stored for five or ten days (thus exposing the emulsion to β-particles emitted by decaying tritium) prior to development.

One hour prior to development, coverslips were removed from the refrigerator and allowed to warm up to dark-room temperature (18°C) in their sealed boxes containing silica gel. They were developed for five minutes in glass staining dishes using freshly prepared, doubly filtered D19 developer (Kodak Ltd., Box 33, Swallowdale Lane, Hemel Hempstead, Hertfordshire) in a dark-room lit with a safelight fitted with a 15 watt bulb and an Ilford 904F filter (Polysciences Inc., Philadelphia, U.S.A.). The developer was then poured off and coverslips rinsed in

100 ml deionised distilled water at 18°C for 30 seconds.

One hundred ml Kodak rapid fixer (Kodak Ltd.) was poured on the coverslips and allowed to act for 10 minutes without agitation. The fixer was poured off and coverslips rinsed briefly in deionised distilled water then in slowly running tap water for at least 30 minutes, following which they were dried in front of a fan for 45-60 minutes and stained by Giemsa's method.

DNA synthesis in cells was assessed by examining 50 cells per coverslip for tritiated thymidine incorporation.

c) Results

Table 10: Exposure of cells to hydroxyurea and evaluation of the degree of synchrony by grain counts of autoradiographs

Concentration of hydroxyurea used in mM	No. of grains per cell (50 cells counted)										Mean grain per cell
	0	0	0	0	0	0	0	0	0	4	
Control	0	0	0	0	0	0	0	0	0	0	
0	0	2	0	0	0	0	0	0	0	0	0.20
	4	0	0	0	0	0	0	0	0	0	
	0	0	0	0	0	0	0	0	0	0	
	0	0	0	0	0	0	0	5	0	0	
	0	0	0	0	0	0	0	0	0	0	
1	4	0	0	0	0	0	3	0	0	0	0.28
	0	1	0	0	0	0	0	0	0	0	
	0	0	0	1	0	0	0	0	0	0	
	8	10	6	1	0	1	3	7	6	6	
	10	0	14	10	5	10	5	8	8	4	
2	4	10	6	8	3	3	9	5	12	2	5.60
	6	5	10	14	1	1	6	11	6	2	
	2	3	0	4	9	0	7	3	2	4	
	23	27	21	20	19	11	5	22	18	22	
	17	16	17	10	11	15	21	16	17	19	
3	12	13	18	21	16	17	18	0	11	14	14.72
	11	10	10	3	13	12	8	24	28	4	
	11	12	10	19	18	21	10	9	5	11	

Cells on coverslips exposed to 1 mM hydroxyurea incorporated tritiated thymidine in about 10% of cells, 2 mM about 80% and 3 mM about 98% of the cells in the population. Autoradiographs showed that cells exposed to 3 mM hydroxyurea incorporated more tritiated thymidine (Fig.15) and the mean grain count per cell exceeded those of cells exposed to 1 mM or 2 mM hydroxyurea (Table 10).

d) Comments

Exposure of serum-starved PK15 cells to 3 mM hydroxyurea synchronised more cells than 1 mM or 2 mM as shown by the incorporation of radioactive thymidine. Preliminary experiments have shown that although 3 mM hydroxyurea was toxic to PK15 cells, CSM still attached to these cells although more avidly than to non-synchronised cells.

4.4.8 Semi-quantitative assessment of the attachment of CSM to synchronised PK15 cells

a) Introduction

Many workers have used different methods to synchronise cell lines and no single method has satisfactorily achieved the same degree of synchrony in all cell lines. Preliminary experiments have shown that 3 mM hydroxyurea although toxic to PK15 cells synchronised about 98% of the cells in a population. This effect on cells did not hinder the attachment of CSM to these cells after 4 hours of growth.

In this present experiment synchronised cells were infected with CSM at different times in the growth cycle of these cells. This sought

to define the period when cells might be refractory to attachment by CSM.

b) Materials and Methods

i) Synchronisation of cells

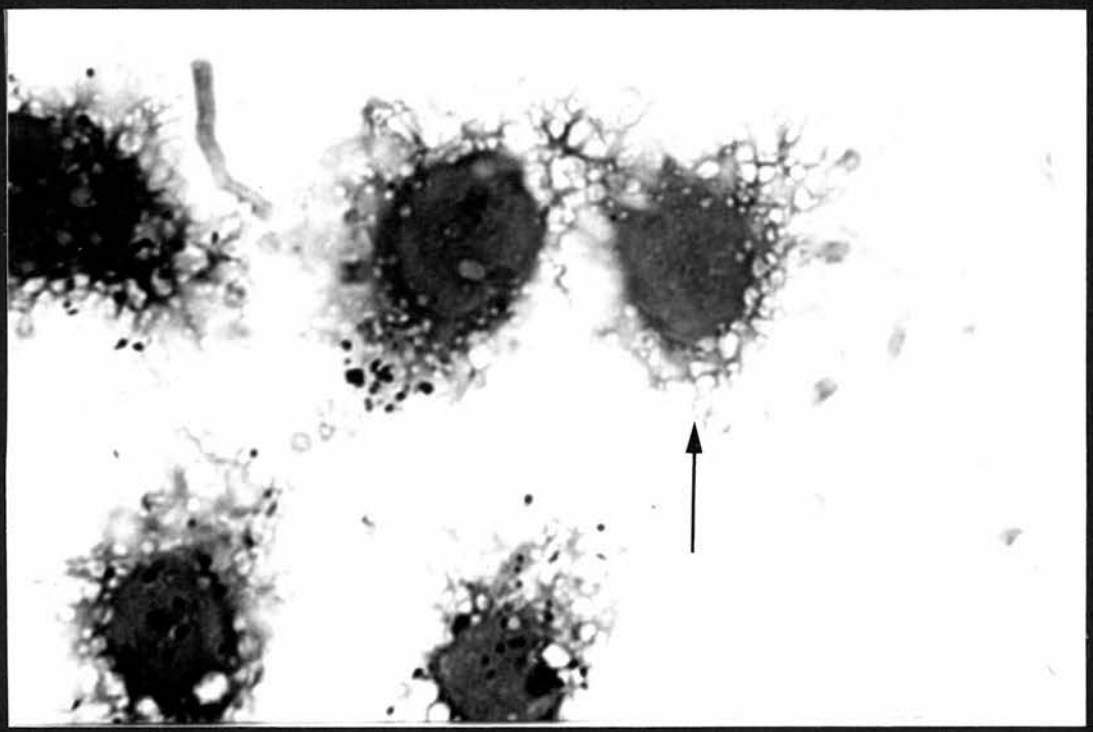
Cells were synchronised by serum-starvation and exposed to 3 mM hydroxyurea as previously described (4.4.7).

ii) Infection of synchronised cells

Synchronised cells were grown on coverslips in 1 ml amounts at a concentration of 1 x 10^3 cells per ml, and at two-hourly intervals from seeding a coverslip monolayer was infected with 1 ml CSM suspension. Coverslips were incubated at 37°C for 1 hour, rinsed in warm PBS, fixed in methanol and stained by Giemsa's method. Untreated cells grown on coverslips were infected at two hourly intervals from seeding and used as controls.

Fig. 15. Autoradiograph of serum-starved PK15 cells after 14 hours exposure to 3 mM hydroxyurea showing incorporation of tritiated thymidine. Note (i) toxicity of hydroxyurea to cells indicated by cytoplasmic vacuoles, and ii) one cell (arrowed) not showing tritiated thymidine incorporation.

Giemsa stain (x 180)



c) Results

Table 11: Semi-quantitative assessment of CSM attachment to synchronised and untreated PK15 cells

Age of cell cultures at time of infection in hours	Intensity of attachment and percentage of cells showing adherent bacteria			
	Synchronised cells		Untreated cells	
2	++++[a]	(98)	-[a]	(0)
4	++++	(99)	-	(0)
6	++++	(98)	-	(0)
8	++++	(98)	-	(0)
10	++++	(98)	+	(55)
12	++++	(97)	+	(63)
14	++++	(96)	++	(71)
16	++++	(96)	++	(88)
18	++++	(96)	+++	(89)
20	++++	(95)	+++	(92)
22	++++	(94)	+++	(93)
24	++++	(95)	+++	(95)

a Predominant cell/bacteria attachment type

\+ = Most of the cells showing less than 10 attached bacteria per cell

++ = Most of the cells showing 11 to 20 attached bacteria per cell

+++ = Most of the cells showingmore than 20attached bacteria per cell

++++ = Most of the cells showing more than 20 or more avidly attached bacteria per cell.

\- = No bacteria attached to cell surface

( ) = Numbers in brackets indicating the percentage of cells showing bacteria attached to cell surface.

The intensity of attachment of CSM to synchronised cells remained similar over the 24 hours of cell growth, with more than 20 bacteria tightly adhering to each cell. There was a small decrease in the number of cells to which bacteria adhered from around 14 hours after cell synchronisation. CSM did not attach to untreated PK15 cells in the first 8 hours of growth and thereafter there was a continued increase in the percentage of cells showing attached bacteria and the intensity of attachment between 10 and 24 hours of growth (Table 11).

d) Comments

CSM attached to synchronised cells and not to untreated cells during the first 8 hours of growth which suggests that the non-attachment of the bacteria during this period is related to the stage in cell growth at the time of infection. However the failure to detect a stage of non-attachment in synchronised cells does not support such a conclusion. It is possible that the cell surface is modified by hydroxyurea resulting in alteration or activation of receptor sites which are otherwise inactive in the early stages of growth of untreated cells.

4.5 Determination of PK15 cell surface receptor for CSM adhesins

4.5.1 Introduction

It has been proposed that when parasites are selective in the cell types which they infect, parasitism may depend on specific recognition systems between host cell and parasite. One concept which

has attracted widespread interest is that specific receptors are expressed on host cell and parasite surfaces and that the interaction of these molecules is essential for infection (Keusch, 1979; Sandin *et al*, 1982; Wyler and Suzuki, 1983). These molecular determinants might include those which mediate initial attachment between host cell and parasite and others which are involved in triggering the cellular events involved in parasite entry.

Investigators have studied host cell-parasite interactions *in vitro* in the search for such molecules and it is now known that certain carbohydrate moeities on both or either host cell and parasite surfaces are involved in these interactions. For example, glucose and mannose residues on *Leishmania donovani* mediate its attachment to hamster peritoneal macrophages (Chang, 1981); N-acetyl glucosamine on mouse fibroblasts (L cells) in the adherence of *Chlamydia psittaci* (Levy, 1979); lipoteichoic acid on group A streptococci on their attachment to oral mucosal cells (Ofek *et al*, 1975, Beachey and Ofek, 1976).

In the following experiments attempts were made to determine the surface components of PK15 cells and CSM that were involved in attachment using a number of lectins. The effects of different salts of heavy metals and chemicals on the attachment of CSM to PK15 were also investigated.

4.5.2 The effects of lectins on the attachment of CSM to PK15 cells

a) Introduction

Lectins are sugar-binding proteins of non-immune origin which sometimes agglutinates certain cells (Goldstein et al, 1980). They have been useful tools in the identification of carbohydrates as part of the cell surface receptors for a variety of microorganisms (Ofek et al, 1978).

A number of studies on lectin-microbe interactions have examined the bindings of whole organisms by purified lectins. Among bacteria found to react with lectins are a variety of Gram-positive and Gram-negative organisms as well as mycoplasmas. Goldstein and Misaki (1970) for example showed that the specific ligand for con A in the agglutination of Mycobacterium bovis was an arabinogalactam found in the cell wall of this organism.

In this experiment, the role of carbohydrate moieties on PK15 cell surfaces in the interaction between these cells and CSM was studied by measuring the ability of three lectins to block this interaction.

b) Materials and Methods

i) Possible toxicity of lectins to PK15 cells and CSM

Soya bean agglutinin (SBA), wheat germ agglutinin (WGA) and concanavalin A (Con A) were tested at concentrations of 10, 30, 50 and 100 μg/ml for evidence of toxicity to PK15 cells and CSM.

PK15 cells

Cells grown in monolayer on coverslips for 24 hours were exposed for 1 hour at 37°C to either 10, or 30, or 50 or 100 μg per ml of each lectin diluted in PBS. Coverslips were rinsed thoroughly in warm PBS, fixed in methanol and stained by Giemsa's method. Cells exposed to PBS for 1 hour at 37°C were stained and used as controls. In a parallel experiment a confluent monolayer of cells was trypsinised and centrifuged at 200 x g for 1 minute, to sediment cells. The supernatant was discarded, the cells resuspended in 2 ml of either lectin or PBS and allowed to stand in a petri-dish at 37°C for 1 hour, after which time the cell suspension was viewed under a light microscope.

CSM

An overnight culture of CSM on a CBA slope was washed off with warm PBS, centrifuged at 4000 x g for 20 minutes, the supernatant discarded and pellet resuspended in 1 ml of each concentration of lectin in PBS. The test tube and contents were incubated at 37°C for 1 hour in a shaking water bath. The organisms were washed three times in PBS at 4000x g for 20 minutes and the pellet resuspended in 1 ml PBS. CSM suspended in PBS was used as a control. A drop of treated and control CSM suspensions were stained by Gram's method and the viability of the CSM confirmed by inoculating 0.1 ml of each suspension onto CBA and incubating microaerophilically in the presence of hydrogen and carbon dioxide for 2 days at 37°C.

ii) Infection of lectin-treated and control cells with treated or control CSM

1) Treatment of CSM with lectin

The method used was similar to that used for the assessment of toxicity of lectin for CSM, but the washed lectin-treated bacteria were resuspended in 9 ml PBS and used for the infection of PK15 cells. Control suspensions prepared similarly without lectin treatment were used at the same density.

2) Infection of PK15 cells in monolayer

Cells grown on coverslips for 24 hours were exposed to 1 ml of either 10, 30, 50 or 100 µg per ml, or to a mixture of the same concentration of each lectin for 1 hour at 37°C. Coverslips were rinsed thoroughly in sterile warm PBS and exposed to 1 ml of either untreated or lectin-treated CSM for 1 hour at 37°C. Coverslips were mixed thoroughly in warm PBS, fixed in methanol and stained by Giemsa's method. Stained untreated PK15 cells that had been infected with untreated CSM were used as control.

3) Infection of lectin-treated PK15 cells in suspension

Confluent monolayers of cells were trypsinised and centrifuged at 200 x g for 1 minute to sediment the cells, the supernatant discarded and the cells resuspended in 1 ml of each concentration of the lectins on PBS for 1 hour at 37°C. The cells were washed three times in warm PBS by centrifugation at 200 x g for 1 minute, resuspended

in cell suspension culture medium as previously used. One ml of this suspension was exposed to either 1 ml of untreated or lectin-treated CSM for 1 hour in a shaking water bath at 37°C. The infected cells were washed three times in warm PBS and smeared on a glass slide. Untreated PK15 cells in suspension exposed to untreated CSM were used as control. The air-dried smears were fixed in methanol and stained by Giemsa's method.

4) Inhibition test for sugar specificity

Some sugars were tested at concentration of 10 mg per ml for their ability to reverse the blocking effect of some lectins on the attachment of CSM to PK15 cells. One ml of 24 hour old cells in suspension was mixed with 1 ml each of lectin, sugar and CSM suspension and incubated for 1 hour at 37°C in a shaking water bath. The methods for washing and staining the cells were similar to those described for lectin-treated cell suspension infected with CSM.

c) Results

Table 12: Toxicity of different concentrations of lectins on PK15 cells and CSM

Lectin concentration in µg per ml		Cells or bacteria exposed to lectins		
		PK15 monolayer	PK15 suspension	CSM
SBA	0	-	-	0
	10	-	-	0
	30	-	-	0
	50	-	-	0
	100	+	+	0
Con A	0	-	-	0
	10	-	-	0
	30	-	-	0
	50	-	-	0
	100	+	+	0
WGA	0	-	-	0
	10	-	-	0
	30	-	-	0
	50	-	-	0
	100	+	+	0

- = No morphological effects of toxicity

+ = Cells exhibiting morphological change

0 = No change in bacterial morphology and bacteria remained viable after exposure for 1 hour.

Table 13a: Attachment of untreated CSM to lectin-treated PK15 cells

Lectin and concentrations used in μg per ml		Cells pre-treated with lectins and exposed to untreated CSM	
		PK15 monolayer	PK15 suspension
Control	0	+++[a]	+++
SBA	10	+++	+++
	30	+++	++
	50	++	++
Con A	10	+++	+++
	30	+++	+++
	50	+++	+++
WGA	10	+++	+++
	30	+++	++
	50	++	++
SBA + Con A + WGA			
	10	+++	+++
	30	+++	++
	50	++	++

a Predominant cell/bacteria attachment type

\+ = Most of the cells showing less than 10 attached bacteria per cell

++ = Most of the cells showing 11 to 20 attached bacteria per cell

+++ = Most of the cells showingmore than 20attached bacteria per cell.

Table 13b: Attachment of treated CSM to lectin-treated PK15 cells

Lectin concentrations used in µg per ml		Cells pretreated with lectins and exposed to treated CSM	
		PK15 monolayer	PK15 suspension
Control	0	+++[a]	+++
SBA	10	+++	+++
	30	++	++
	50	++	+
Con A	10	+++	+++
	30	+++	+++
	50	+++	+++
WGA	10	+++	+++
	30	++	++
	50	++	+
SBA + Con A + WGA			
	10	+++	+++
	30	++	++
	50	++	+

a Predominant cell/bacteria attachment type

\+ = Most of the cells showing less than 10 attached bacteria per cell

++ = Most of the cells showing 11 to 20 attached bacteria per cell

+++ = Most of the cells showing more than 20 attached bacteria per cell.

Table 14: Attachment of CSM to PK15 cell suspension in the presence of both sugars and lectins

Lectin (50μg/ml) and Sugar (10mg/ml) used	Degree of bacterial attachment
Control 0	+++
SBA	+[a]
SBA + Nacetyl-D-galactosamine	+++
SBA + D-Mannose	++
SBA + D-galactose	++
SBA + D-glucose	++
WGA	+
WGA + Nacetyl glucosamine	+++
WGA + D-galactose	++
WGA + D-glucose	++
WGA + D-Mannose	++

a Predominant cell/bacteria attachment type:

= = Most of the cells showing less than 10 attached bacteria per cell

++ = Most of the cells showing 11 to 20 attached bacteria per cell

+++ = Most of the cells showingmore than 20attached bacteria per cell.

All 3 lectins at concentration of 100µg per ml showed cytotoxic effects on PK15 cells in monolayer or suspension, as evidenced by the appearance of cytoplasmic vacuoles in Giemsa-stained coverslip monolayers, and shrinkage of unstained cells in suspension. The lectins had no effect on the morphology or viability of CSM at any concentration (Table 12).

SBA and WGA at 50µg per ml either separately or together partially prevented the attachment of either treated or untreated CSM to treated PK15 cells (Table 13a and 13b). Those effects were more marked in treated cells exposed to treated bacteria (Table 13b). At 10µg per ml these lectins did not prevent attachment of bacteria to cells. Con A did not have any inhibitory effect on attachment at the concentrations tested.

Nacetyl-D-galactosamine reversed the inhibitory effect of SBA on the attachment of CSM to PK15 cells as did Nacetyl-glucosamine on the effect of WGA. The other sugars reversed the effect of SBA or WGA to a lesser extent than Nacetyl-D-galactosamine or Nacetyl glucosamine respectively (Table 14).

d) Comments

The quantitative differences in attachment of CSM to PK15 cells in these experiments were not clear cut. The partial inhibition of the attachment of CSM to PK15 cells by both SBA and WGA showed that there may be multiple receptor sites on PK15 cell surfaces for CSM adhesins. Rajasekhar (1981) reported that 50µg per ml con A partially prevented the attachment of CSM to PK_{pi} cells. These cells, which are Stice pig

kidney cells persistently infected with Newcastle Disease Virus, have altered cell surface membranes (Fraser et al, 1976), and this may explain why con A at the same concentration did not show any inhibitory effect on the attachment of CSM to PK15 cells.

Since N acetyl-D-galactosamine and N acetyl glucosamine reversed the inhibitory effects of SBA and WGA respectively, this supports the specificity of the inhibition and indicates that these sugar moeities on the cell surfaces play a part in the attachment of CSM to PK15 cells.

4.5.3 The effect of CSM antiserum, enzymes, salts of metals and hydroxyurea on attachment of CSM to PK15 cells.

a) Introduction

Many workers have studied the effects of environmental, host and bacterial factors on the attachment of pathogenic bacteria to cell surfaces and it has been shown that specific anti-bacterial serum may inhibit this reaction. For example, in the adhesion of Klebsiella pneumoniae to rat bladder epithelial cells (Fader et al, 1979), in the adhesion of Bordetella bronchiseptica to pig nasal epithelial cells (Yokomizo and Shimizu, 1979). In contrast to these observations, the enhancement of bacterial adhesion to the host cell by specific antiserum has been reported by Hale and Bonventre (1979) in the adhesion of Shigella flexneri 2a to Henle intestinal epithelial cells and more recently in the adhesion of Leptospira interrogans serovar copenhageni L45 to mouse fibroblast cells - L929 (Vinh et al, 1984). In a study of the adhesion of opsonised bacteria by phagocytic cells

van Oss (1978) suggested that the coating of bacterial surfaces with immune serum may render them more hydrophobic thus promoting close contact with the host cell membrane.

Inhibitors of cellular DNA synthesis such as cycloheximide and methotrexate, prevented the attachment of Treponema pallidium to baby rabbit genital organ cells (Wong et al, 1983). The adhesion of Yersinia pseudotuberculosis to the surface of HeLa cells was not enhanced by the presence of calcium and magnesium salts in the medium (Brunius and Bölin, 1983).

In this experiment, the effects of various agents on attachment of CSM to PK15 cells were investigated. PK15 cells were pretreated with either specific CSM antiserum, salts of heavy metals, hydroxyurea or enzyme and then infected with CSM to assess the extent to which pretreatment of cells with any of these agents hindered or enhanced attachment of CSM to the cells.

b) Materials and Methods

i) Pretreatment of cells with CSM antiserum and infection with CSM

One-day-old coverslip cultures of PK15 cells were refed with maintenance medium containing 1% calf serum and 1% rabbit anti-CSM (strain 253/72) serum prepared by the method described in 2.5.6. After 2 hours of incubation at 37°C the pretreated cells were rinsed in two changes of PBS to remove traces of serum, overlaid with 1 ml of CSM suspension and incubated at 37°C for 2 hours. Coverslips of cells not treated with CSM anti-serum were also inoculated with 1 ml of bacterial suspension and used as control. Both the treated and control coverslips

were stained by Giemsa's method.

ii) Pretreatment of cells with salts of heavy metals or hydroxyurea and infection with CSM

Salts of heavy metals or hydroxyurea prepared in PBS were used at a concentration of 1 mM. One-day-old coverslip monolayers of PK15 cells were exposed to either 1 ml of each heavy metal salt or hydroxyurea for 1 hour at 37°C. The coverslips were rinsed twice in PBS and infected with 1 ml of CSM suspension and incubated at 37°C for 2 hours. Coverslips not treated with any heavy metal salt or hydroxyurea were infected with 1 ml of bacterial suspension and used as controls. All coverslips were stained by Giemsa's method after appropriate washing and fixation.

iii) Pretreatment of cells with enzymes and infection with CSM

One-day-old PK15 cells grown in medical flats were rinsed in warm PBS and detached from the glass with a sterile rubber 'policeman'. The cells were exposed to either 0.1% or 1% of trypsin or of receptor destroying enzyme (RDE) in a centrifuge tube for 30 minutes in a shaking water bath at 37°C. The cells were centrifuged at 200 x g for 1 minute to sediment the cells, the supernatant discarded and the cells resuspended in maintenance medium. To 1 ml of treated cells was added 1 ml of CSM suspension and the mixture incubated in a shaking water bath at 37°C for 2 hours. The infected cells were washed three times in PBS at 200 x g for 1 minute and resuspended in 1 ml of PBS. A drop of this washed infected cells was air-dried on a glass-slide, fixed in methanol, and stained by Giemsa's method. Cells that were not exposed

to any enzymes were infected with CSM and used as controls.

c) Results

Table 15: Attachment of CSM to treated and control PK15 cells

Agent used for pretreatment of PK15 cells	Intensity of attachment
None	+++
CSM-antiserum	-
Ferric chloride	+++
Ferrous chloride	+++
Calcium chloride	+++
Magnesium chloride	+++
Manganese chloride	+++
Potassium chloride	+++
Sodium chloride	+++
Zinc chloride	+++
Hydroxyurea	++++
Trypsin 0.1%	+++
1.0%	+++
R.D.E.* 0.1%	+++
1.0%	+++

\- = No bacterial attachment

+++ = Bacterial attachment

* Wellcome Research Laboratories, Beckenham, England.

None of the heavy metal salts or enzymes prevented or enhanced the attachment of CSM to PK15 cells (Table 15). CSM antiserum prevented the attachment of CSM to PK15 cells while hydroxyurea enhanced it. Untreated infected control cells showed the normal attachment of CSM.

d) Comments

In this experiment, no heavy metal salt showed any effect on the attachment of CSM to PK15 cells. It is not known whether the concentration of salt used was too low to effect any change and possibly further work might justify the use of a wider range of concentrations of more likely candidate salts. Sugarman (1980) worked with buccal cells and strains of Enterobacteriaceae but used 0.3 mM of these salts and reported enhancement of attachment by zinc and iron salts. It appears therefore that, the concentration and type of metallic salt can both effect a change in attachment and all cell types may not behave in the same way. Trypsin and RDE at concentrations of 0.1% and 1% did not prevent or enhance the attachment of CSM to PK15 cells. RDE specifically attacks neuraminic acid-containing mucopolysaccharide of cell surface receptors. Organisms that attach to cell surfaces via these receptors cannot attach after RDE treatment. RDE had no effect on receptor sites for CSM attachment. This result suggests that the receptor sites on PK15 cells may not contain neuraminic acid residues.

Previous experiments with 1 mM hydroxyurea (4.4.6) showed that this concentration hydroxyurea did not synchronise PK15 cells after 14 hours of exposure but showed the same enhanced pattern of CSM

attachment as cells synchronised with 3 mM hydroxyurea. Since cells treated with 1 mM hydroxyurea for 1 hour showed this enhanced bacterial attachment when exposed to CSM, it appears that this effect is due to hydroxyurea modifying the receptor sites on the cell surfaces and not an effect of synchronisation of those cells .

CSM antiserum used in this experiment was prepared against live bacterial cells and probably contains antibodies to most of the bacterial surface components including flagella. The specific nature of CSM attachment to PK15 cells is further evidenced by the ability of this antiserum to prevent attachment. This result confirms the observation of Rajasekhar (1981) who demonstrated that either pretreatment or the presence of specific anti-CSM antisera in the supernatant fluid at the time of infection completely blocks adherence of CSM to PK15 cells. In this extended series of experiments, searching for mechanisms that might elucidate the receptor sites anti-CSM antisera has proved the only factor capable of completely inhibiting attachment.

4.6 Comparison of the attachment of CSM and E. coli to pig brush borders

4.6.1 Introduction

Attachment of enteropathogenic bacteria to the mucosal surface of intestinal epithelial cells is an important determinant of virulence for some enteric organisms (Jones and Freter, 1976; Isaacson et al, 1978). The adherence of pathogenic E. coli appears to regulate the ability of the organism to colonise the small bowel by allowing the

pathogens to replicate while resisting the possible clearing action of intestinal peristalsis (Dixon, 1960).

The adhesive properties of K88 (Jones and Rutter, 1972), K99 and 987P (Moon et al, 1980) pilus antigens of E. coli allow bacterial attachment to piglet intestinal epithelium. K88-positive E. coli adhere to brush borders prepared from small intestinal epithelial cells from many but not all pigs (Sellwood et al, 1975; Snodgrass et al, 1981). Susceptibility to diarrhoea caused by both natural and experimental infection with K88 positive E. coli is limited to piglets of adherent phenotype (Rutter et al, 1975; Sellwood, 1979).

When gnotobiotic pigs were exposed to CSM colonisation took place but there was very little evidence of marked attachment to the epithelium and only minimal cell penetration. It was not known whether the pigs used were typical of all pigs or if other pigs might respond differently. Gnotobiotic experiments are expensive and time consuming which limits the number of such experiments that can be undertaken and the number of genotypes that can be tested for susceptibility. For these reasons, an alternative approach was the examination of attachment of CSM to brush border cells in vitro. In order to assess attachment and to ensure that at least 2 of the known pig intestinal cell genotypes had been employed, attachment tests were performed with $K88^{+}$ and $K88^{-}$ E. coli and CSM.

4.6.2 Materials and Methods

i) Sources of pigs and collection of samples

Intestinal samples used for the preparation of brush borders were collected from freshly slaughtered pigs, derived from a number of sources. Samples were obtained on different occasions from a total of 38 pigs slaughtered at Gorgie Abattoir, Edinburgh, tissue from 2 further animals were obtained one from Animal Breeding Research Organisation, Edinburgh and one from Easter Howgate Farm, Midlothian. Segments of mid ileum approximately 7 cm x 4 cm were excised from freshly killed pigs and washed free of contents with chilled 0.15M sodium chloride and transported to the laboratory in this solution.

ii) Preparation of brush borders from intestinal segments

The technique of Sellwood et al, (1975) was used to prepare brush borders from intestinal segments.

In the laboratory the saline solution was replaced by a solution containing 0.96M sodium chloride, 0.008M potassium hydrogen phosphate, 0.0056M sodium hydrogen phosphate, 0.0015M potassium chloride and 0.01M ethylenediaminetetraacetic acid (EDTA) pH 6.8 (Evans et al, 1971) and the segment of intestine left in this solution for 15 minutes at room temperature.

All further steps were performed with fluid chilled to 4°C except where stated otherwise. The mucosal surface of the specimen was scraped with a scalpel blade into 5 ml of a solution similar to the above except that it contained 0.3M sucrose in place of EDTA.

This cell suspension was homogenised in a Teflon topped tissue grinder (Jencons Scientific Ltd., Hemel Hempstead, England,- Catalogue no. 104/8/95 - with a gap of 0.76 mm - 0.126 mm cell round clearance) by moving the pestle up and down while it rotated at about 1000 r.p.m. (Sellwood et al, 1975). The homogenate was centrifuged at 1200 x g for 10 minutes and the pellet resuspended in 5 ml 0.005M EDTA pH 7.4. (adjusted with 0.5M sodium carbonate). Homogenisation was repeated and the suspension centrifuged at 300 x g for 4 minutes. The pellet was then washed in Krebs-Henseleit buffer pH 7.4 containing 0.12M sodium chloride, 0.014M potassium chloride, 0.25M sodium bicarbonate and 0.001M potassium hydrogen phosphate and finally resuspended in this buffer. Brush border preparations were used on the day of preparation for adhesion assays.

iii) Preparation of bacterial inocula

The methods for the preparation E. coli and CSM inocula have been described in 2.5.3.

iv) Brush border adhesion test

A volume of 0.1Ml of brush border suspension was added to 0.1Ml of either E. coli or CSM suspension in a small screw capped vial (1 cm x 3.5 cm) and the vial with contents incubated in a shaking water bath at 37°C for 45 minutes. One drop of each of the three brush border-bacteria reaction mixtures was dried on a microscope slide, fixed in methanol and stained by Giemsa's method.

v) PK15 positive adhesion test for CSM

A 24-hour-old coverslip monolayer of PK15 cells was infected with 1 ml of a day-old CSM culture suspended in warm PBS as described previously. The infected coverslip monolayer was incubated at 37°C for 45 minutes, washed with warm PBS fixed in methanol and stained by Giemsa's method.

4.6.3 Results

i) Adhesion of E. coli to brush borders

$K88^-$ E. coli did not adhere to brush borders prepared from any pig.

All brush borders prepared from 7 pigs showed adhesion of $K88^+$ E. coli (more than 10 bacteria per brush border), (Fig. 16) while those prepared from 23 pigs showed no adhesion (Fig. 17) Brush borders prepared from 10 pigs showed variable and limited adherence of $K88^+$ E. coli (less than 10 bacteria per brush border in about 7 out of 20 brush borders) Table 16.

ii) Adhesion of CSM to brush borders

Adhesion of CSM to brush borders from each of the 40 pigs was scanty compared with adhesion of $K88^+$ E. coli to receptive brush borders. CSM adhered to both receptive and non-receptive brush borders with similar intensity. CSM adhered to brush border surface and all counts were performed on attachment to this surface (Fig. 18).

Fig. 16. Adherent pig brush border after 45 minutes of exposure to K88$^+$ *E. coli*. Bacteria are attached to the convex brush border (specific attachment).

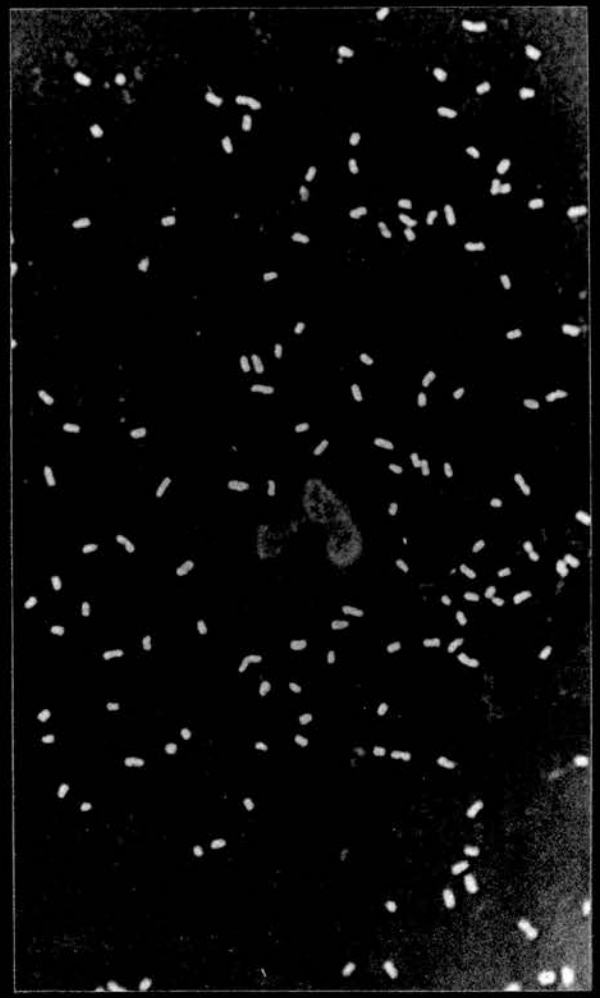
Giemsa stain (x 180)

Fig. 17. Non-adherent pig brush border 45 minutes after exposure to K88$^+$ *E. coli*. There is no attachment of bacteria.

Giemsa stain (x 180)

Fig. 18. Non-adherent pig brush border 45 minutes after exposure to CSM. Only occasional bacteria are adherent.

Giemsa stain (x 180)



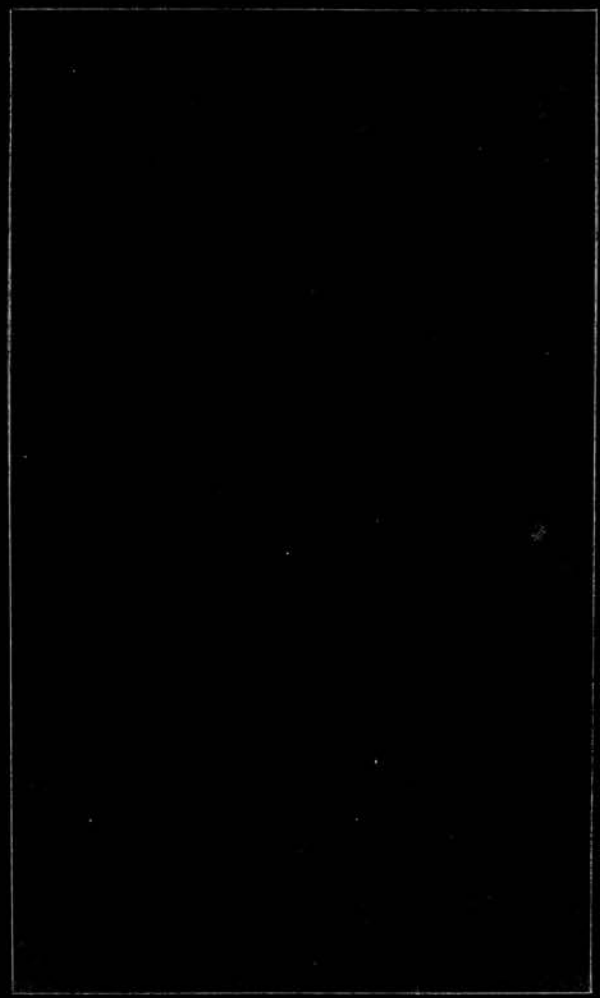


Table 16: Attachment of K88$^+$ *E. coli* or CSM to pig brush borders

	Pig/Sample number	Mean number of adherent organisms per brush border (20 brush borders counted)			
		K88$^+$ *E. coli*	CSM		K88$^-$ *E. coli*
Adherent phenotype	1	17.50	3.50		0
	2	13.70	3.60		0
	3	14.55	3.15		0
	4	15.25	3.00		0
	5	18.40	3.25		0
	6	18.25	3.70		0
	7	15.50	3.20	$\bar{x}$ = 3.34	0
Varied phenotye	8	4.00	3.60		0
	9	5.95	3.50		0
	10	6.25	3.60		0
	11	5.65	3.15		0
	12	5.20	3.00		0
	13	4.20	3.40		0
	14	3.40	3.60		0
	15	4.30	3.15		0
	16	4.00	3.50		0
	17	6.20	3.20	$\bar{x}$ = 3.37	0
Non-adherent phenotype	18	0	3.10		0
	19	0	3.40		0
	20	0	3.55		0
	21	0	3.60		0
	22	0	3.40		0
	23	0	3.10		0
	24	0	3.20		0
	25	0	3.40		0
	26	0	3.70		0
	27	0	3.00		0
	28	0	3.40		0
	29	0	3.20		0
	30	0	3.50		0
	31	0	3.40		0
	32	0	3.50		0
	33	0	3.45		0
	34	0	3.20		0
	35	0	3.40		0
	36	0	3.20		0
	37	0	3.40		0
	38	0	3.65		0
	39	0	3.75		0
	40	0	3.20	$\bar{x}$ = 3.38	0

iii) Adhesion of CSM to PK15 cells

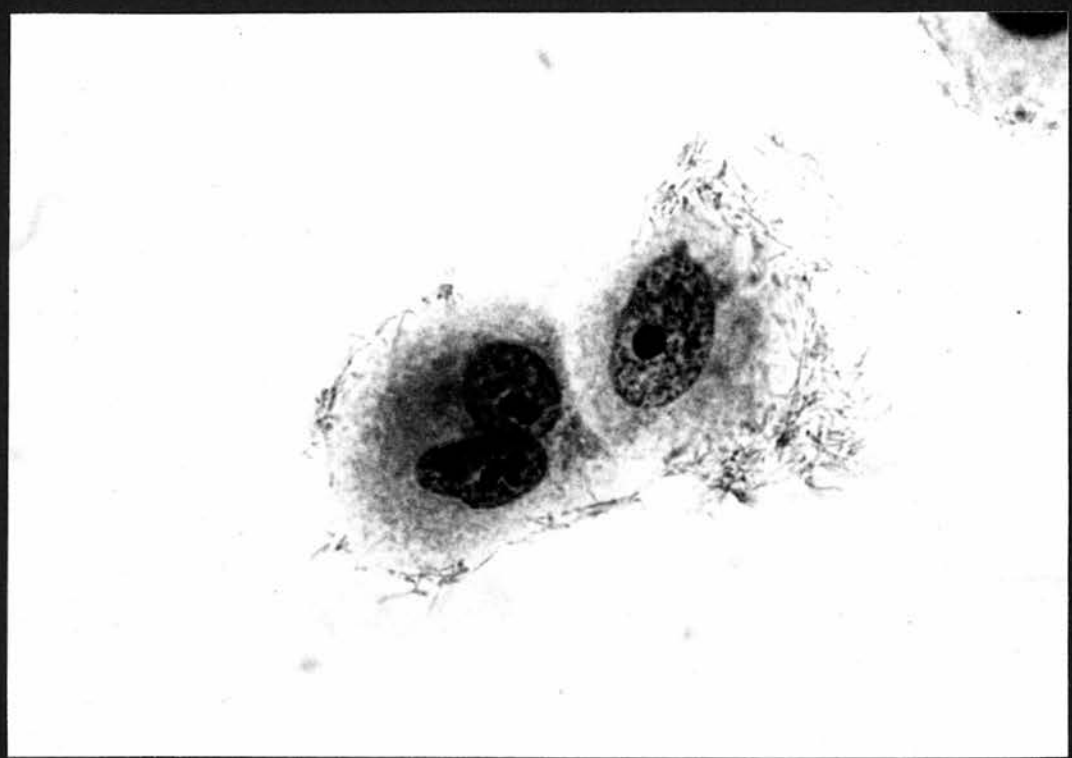
Giemsa-stained preparations of coverslip monolayers of PK15 cells infected with CSM for 45 minutes showed the normal dense attachment of bacteria to the periphery of the cell (Fig. 19).

4.6.4 Comments

The inocula of CSM used in these experiments contained between 10.18 $\log_{10}$ and 11.60 $\log_{10}$ organisms per ml, while that of E. coli was between 8.48 $\log_{10}$ and 9.60 $\log_{10}$ organisms per ml. The difference in the numbers of bacteria should not have made a significant difference to the results and would in any case favour CSM attachment. These results suggest that i) the receptor sites for the adhesion of $K88^+$ E.coli to brush borders are different from the sites for CSM adhesion since CSM adheres to both K88 adherent and non-adherent brush borders. ii) the receptor sites for CSM adhesion on pig brush borders or PK15 cells are either different or considerably less numerous in the former because adherence to brush borders is minimal in comparison with PK15 cells. iii) the attachment of CSM to intestinal epithelial cells of different pigs is not likely to be materially different. These results therefore suggest that further gnotobiotic experiments using genetically different pigs would not be justified at the moment. The number of pigs examined was small, however the widespread occurrence of the proliferative diseases and the number of animals that can be affected especially in outbreaks of PHE suggest that a reasonable proportion of the pig population is likely to be susceptible.

Fig. 19. PK15 cells 45 minutes after exposure to CSM. Note extensive bacterial attachment to the periphery of the cells.

Giemsa stain (x 180)



4.7 Discussion

The six strains of CSM examined showed a similar intensity and pattern of attachment to PK15 cells. This attachment was transitory and reached a maximum around 2 to 4 hours post infection and thereafter attached extracellular organisms could only be demonstrated for 10 to 12 hours.

A comparative evaluation of bacterial attachment to PK15, MDBK and Int 407 cell types observed during the peak period (2 to 4 hours) indicated that greater numbers of bacteria attached to PK15 cells than to the other two cell lines which showed similar intensity of attachment by CSM. In Int 407 cells there was increased attachment after 6 hours which continued till 48 hours but the organisms were uniformly dispersed over the cell surface and showed no particular preference for the periphery of the cell. Subsequent tests showed that these organisms were demonstrable on the cell surface in decreasing numbers for 13 days. This pattern of initial attachment to cells is similar to that reported by Rajasekhar (1981) for air-inactivated CSM. It appears that CSM remains on the cell surface for a long period or is not internalised in Int 407 cells, this adherence differs from that seen in PK15 or MDBK cells. The slow nature of this process in Int 407 cells may lead to air-inactivation of extracellular organisms and this results in the uniformly dispersed pattern of attachment. Negatively stained preparations of CSM in culture (Rajasekhar, 1981) showed that 24 hour-old bacteria had a rough scaly outer surface or cell coat, while 4 day-old cells had smooth surfaces. It is not clear whether this difference in cell surface appearance is related to the varied intensity of attachment

even in PK15 cells infected with CSM of these ages. The possibility of the involvement of cell surface structures in the attachment of CSM to cells is more convincing than attachment by flagella. Scanning or transmission electron micrographs of cells infected with CSM showed that bacteria were aligned horizontally or tangentially on the cell surface and in the latter case there was never any evidence of a flagellum interposed between cell and bacterial envelop. These observations suggest that the flagellar antigens do not specifically mediate adherence.

Although factors such as i) the concentration of bacterial inoculum ii) cell to cell contact, and iii) stage in cell growth at the time of infection, have been shown to play a part in the refractory nature of some cells in a population to the attachment of CSM, about 2% of the cells still do not show any bacterial attachment. Since different cells in an asynchronous population have different generation times it may be that these refractory cells are, or remain, immature and do not develop receptor sites for CSM attachment.

The bacterial inoculum should contain 10 $\log_{10}$ organisms per ml to achieve attachment to 95% of the cells in the PK15 cell infection system. At lower bacterial concentrations there was both a reduction in the number of organisms attached to cells and the number of cells showing attached bacteria. This result should be expressed since at higher bacterial concentration the bacteria per cell ratio is clearly higher than at lower CSM concentrations.

Experiments with suspension cultures of PK15 cells appeared to indicate that cell to cell contact in monolayers may contribute to the proportion of apparently non-receptive cells. Almost all the cells in suspension showed bacterial attachment and this may be due to the

surface of cells in such culture not being protected physically from exposure to bacteria.

Cells did not show bacterial attachment after 4 to 8 hours of growth but maximum attachment was attained after around 20-24 hours of growth. There was a small decrease in the number of cells showing attachment after exposure at 2 and 3 days of growth. After 2 days the monolayer reaches confluence and cell to cell contact and a high cell to bacteria ratio may start to affect attachment.

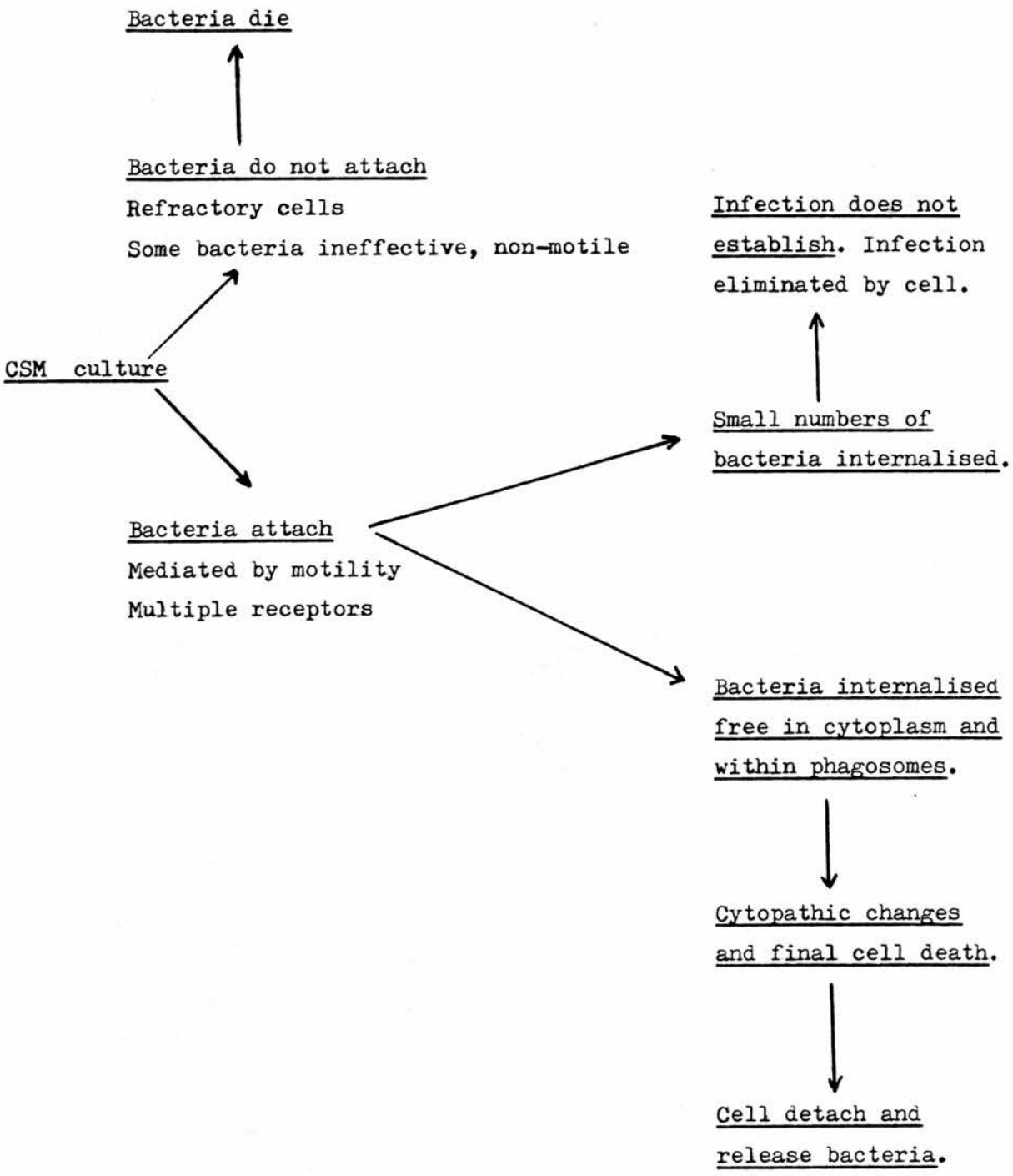
Synchronisation of PK15 cells was undertaken to assess whether the cells of a completely synchronised population would show periods of non-attachment for CSM during some stages of growth. The exposure of serum-starved cells to hydroxyurea synchronised the majority of cells in the population, however it was not possible to detect differences in attachment subsequent to the attempted synchronisation. It has been shown that after around 4 - 8 hours of PK15 cell growth CSM did not attach to the cells which appears to indicate that the stage in cell growth affected attachment of the bacteria to the cells. Since all the cells in a population do not enter the different stages of cell division at the same time it does not seem likely that it is a specific stage in the cell cycle that is refractory to attachment but rather that it is some more general feature coupled to activity of the cell.

The pattern of attachment of CSM to PK15 cells exposed to hydroxyurea was denser than untreated cells. Experiments with cells exposed to 1 mM hydroxyurea for 1 hour showed that hydroxyurea exerted this effect on attachment at the level of receptor sites on the cell surface, since this concentration of hydroxyurea did not synchronise

cells. It is also possible that modification of the cell surface by hydroxyurea alters the receptor sites which appear inactive in the early stages of growth of untreated cells.

The complexity of the process of CSM attachment to PK15 cells is illustrated by the discussion of the results obtained in this study. It appeared that N acetyl-D-galactosamine and N acetyl-glucosamine sugar moeities on the cell surfaces play some role in the attachment of the organisms to cells but these only comprise a minority of the receptors which allow attachment of CSM to PK15 cells. A diagram that summarises the possible interactions of CSM with tissue culture cells is given in Fig. 20.

Fig. 20 Summary of CSM interaction with tissue culture cells.



CHAPTER 5.

Chapter 5

Quantitative assessment of intracellular multiplication of CSM in cultured cells

5.1 General Introduction

5.2 Assessment of intracellular multiplication of CSM in PK15, MDBK and Int 407 cells by the surface viable count method

5.2.1 Vigorous washing as a means of eliminating extracellular CSM from infected cell monolayers

a) Materials and Methods

b) Results

c) Comments

5.2.2 Viable counts from CSM-infected PK15 cells disrupted by homogenisation after the elimination of extracellular bacteria

a) Introduction

b) Materials and Methods

c) Results

d) Comments

5.2.3 Viable counts of infected PK15 cells lysed by Nonidet P40 after the elimination of extracellular bacteria

a) Introduction

b) Assessment of lysis of PK15 cells by Nonidet P40

c) Results

d) Growth of CSM in the presence of Nonidet P40

e) Results

f) Production of cell lysis by NP40 and viable counts of the lysate

g) Results

h) Comments

5.2.4 Total viable count of 'unwashed' infected cells lysed with Nonidet P40.

a) Introduction

b) Materials and Methods

c) Results

d) Comments

5.3 Assessment of intracellular multiplication by immunofluorescence staining

5.3.1 Introduction

5.3.2 Materials and Methods

5.3.3 Results

5.3.4 Comments

5.4 Discussion

Chapter 5

Quantitative assessment of intracellular multiplication of CSM in cultured cells

5.1 General Introduction

Cell invasion is an important step in the pathogenesis of some bacterial species (Kihlström, 1977; Schiemann and Devenish, 1982; Okumara et al, 1983). The exposure of cells to bacteria sometimes results in bacterial penetration with or without subsequent intracellular multiplication (Kihlström, 1977; Egwu and Eveland, 1979; Brunius, 1980).

A variety of techniques have been used to assess the intracellular growth of bacteria in experimental cell infections. Several methods including bacterial counts on stained preparations, viable counts of "cell-cultured" bacteria and immunofluorescence staining have been found useful in assessing the intracellular growth of bacteria in cell culture systems. Although these methods when used individually do not necessarily provide conclusive evidence of the persistence of intracellular organisms, a better picture emerges when the infection is studied by a number of procedures.

Counts of bacteria in stained preparations do not appear to be satisfactory as the method fails to distinguish adequately intracellular bacteria from those located on the cell surfaces.

Immunofluorescence staining has been used for the demonstration and assessment of intracellular multiplication of bacteria in infected cell cultures (Horwitz and Silverstein, 1980, 1981; Daisy et al, 1981; Holden et al, 1984). Unfortunately, this technique despite its high degree of specificity, does not clearly distinguish between viable or

dead bacteria and degraded bacterial antigens. There may also be difficulty in clearly distinguishing between intra- and extracellular organisms. Workers in the past have relied on the inability of antiserum to penetrate the outer membrane of unfixed cells to demonstrate bacteria bound to the cell surface. Later, it was shown that prefixation of cells in formalin prevents the penetration of antibodies through cell membranes and such a procedure has been found particularly useful in locating viral antigens on cell surfaces (Fraser, 1976). Fixation of infected cells in acetone allows demonstration of intracellular organisms because this treatment is believed to facilitate the passage of antibodies through the cell membrane (Nairn, 1976).

Assessment of intracellular bacterial multiplication by the viable count method appears in some circumstances to be the most accurate method. Since only live organisms are recovered the information obtained is not confused by the presence of phagocytosed dead bacteria or non-viable intracellular forms. This method is not without its pitfalls, not the least being extracellular multiplication in the fluid phase of the cell culture system. Some have attempted to overcome this problem by the use of antibiotics in the culture media; however, as discussed previously, the use of antibiotics to control extracellular bacteria (Kihlström, 1977; Wong et al, 1980; Daisy et al, 1981; Rajasekhar, 1981) is likely to introduce further problems of interpretation.

In this chapter the assessment of intracellular growth of CSM in PK15, MDBK and Int 407 cells was carried out by viable counts and by immunofluorescence staining of the bacteria.

5.2 Assessment of intracellular multiplication of CSM in PK15, MDBK and Int 407 cells by the surface viable count method

The method of assessing intracellular multiplication of pathogenic bacteria in infected cell cultures by viable counts is a three-step procedure namely: i) elimination of extracellular bacteria by the use of appropriate methods ii) lysis of infected cells to release intracellular bacteria and iii) isolation of the organisms on suitable bacteriological media.

Attempts by Rajasekhar (1981) to eliminate extracellular bacteria by the use of streptomycin or gentamycin resulted in bacterial sterilisation of the infected cell culture. For this reason an alternative method was chosen whereby extracellular organisms were removed by vigorous washing of the infected cell monolayers with warm PBS. The first experiment (5.2.1) sought to assess the extent to which such a procedure eliminated extracellular bacteria. Subsequent experiments assessed intracellular multiplication of bacteria in infected cell cultures freed of extracellular bacteria by washing.

5.2.1 Vigorous washing as a means of eliminating extracellular CSM from infected cell monolayers

a) Materials and Methods

PK15, MDBK and Int 407 cells were grown in 4 oz medical flats for 24 hours, the supernatant fluid discarded and the monolayer overlaid with 10 ml of a 24 hour culture of CSM suspended in warm MEM. Infected cells were incubated at 37°C for 24 hours, the supernatant fluid

discarded and the monolayer washed with ten changes of 10 ml of warm PBS. This washing was carried out by sharply tilting the medical flat 20 times for each change of PBS, so that the fluid washed and then drained from the monolayer. The number of organisms in the inoculum and recovered from the last rinse was determined by viable counts.

b) Results

Bacteria could not be recovered from the last wash (≤ 50 organisms/ml) from infected PK15 cells but were recovered from those from infected Int 407 cells (Table 17). A viable count was not carried out on the last rinse from infected MDBK cells because the vigorous washing detached the monolayer from the glass. The other cell monolayers (PK15 and Int 407) withstood the washing process without appreciable loss of cells.

c) Comments

Vigorous washing appears to be a promising means of eliminating extracellular bacteria from infected preformed monolayers of PK15 but not from either MDBK or Int 407 cells. There are two possible problems with this method, one is that of reproducibility, since the washing is carried out manually, the second exists with some cell lines only and is detachment of infected cells from the glass.

In view of these encouraging results, the assessment of intracellular growth of CSM in PK15 cells was undertaken after washing the cells to remove extracellular bacteria. Because of the difficulty of standardising the technique confirmation that the last wash was free of CSM was obtained at each stage and the relative integrity of the monolayer

Table 17: The recovery of CSM from the last of 10 rinses from tissue culture cells infected for 24 hours

Cell line	Bacterial counts as $\log_{10}$ organisms per ml	
	Inoculum	Last rinse
PK 15	8.40	NR
MDBK	8.40	ND
Int 407	8.40	4.10

NR = No organisms recovered

ND = Not examined.

was confirmed by microscopy. The cell-associated growth of CSM in MDBK or Int 407 cells was assessed by total viable counts from infected cells (5.2.4) and by the immunofluorescence staining method (5.3).

5.2.2 Viable counts from CSM infected PK15 cells disrupted by homogenisation after the elimination of extracellular bacteria

a) Introduction

The disintegration of infected cell cultures to release intracellular bacteria is an important step in the procedures used to quantify intracellular multiplication of pathogenic microorganisms. Several methods have been used by different workers for this purpose including mechanical disruption in tissue grinders or homogenisers, ultrasonic disruption of infected cells or cell lysis by chemicals such as Nonidet P40.

In this experiment infected cells were disrupted in an overhead motor-driven homogeniser (MSE) and the number of organisms in the homogenate determined by viable count method.

b) Materials and Methods

PK15 cells were grown in medical flats for 24 hours, the supernatant fluid discarded and the monolayer overlaid with 10 ml of a 24-hour culture of CSM suspended in warm MEM. Infected cells were incubated at 37°C and each day for 10 days an infected monolayer in one medical flat was washed to eliminate extracellular bacteria as previously described in 5.2.1. The washed monolayer was detached from the glass with 10 ml STV

at 37°C and the cells deposited by centrifugation at 200 x g for 1 minute. The cell pellet was resuspended in 10 ml MEM and the mixture was cooled in ice and homogenised at maximum speed (13,000 rpm) in an overhead blender (Blending Assembly for 1 oz (30 ml) universal container, Cat. No. 7700-A, MSE, Cawley, Sussex, England) for 1 minute. The number of organisms in the homogenate was determined by surface viable count.

c) Results

Extracellular bacteria were eliminated from infected PK15 cells by vigorous washing throughout the period of this experiment (Table 18). The number of organisms recovered from the homogenate of infected cells increased to 4 days post-inoculation, thereafter a 3-day period of relative stability ensued followed by a decline until organisms were no longer recovered after 9 days of infection. Examination of the homogenate under the light microscope revealed that about 6 - 7% of the cells were not lysed by this method.

d) Comments

These results provide further evidence that CSM multiplies intracellularly during its interaction with PK15 cells. Although the precise mechanisms involved in the intracellular multiplication of CSM are not known the evidence suggests that the presence of organisms is closely associated with the survival of the infected cells. It will be recalled (Chapter 3) that from 4 days after infection there was a marked increase in the cytopathic changes produced by CSM in PK15 cells ending in total destruction of the cell monolayer after 10 days.

Table 18: Viable counts of CSM from lysed infected PK15 cells after the elimination of extracellular bacteria

Time after infection in days	Last rinse of infected monolayer	Homogenate of infected monolayer
1	NR	2.44*
2	NR	2.80
3	NR	3.10
4	NR	4.92
5	NR	4.80
6	NR	4.63
7	NR	3.51
8	NR	2.10
9	NR	NR
10	NR	NR

* = Count of CSM as $\log_{10}$ organisms per ml (corrected to two decimal places)

NR = No organisms recovered

Infecting dose = 10.44 $\log_{10}$ organisms per ml.

The increased recovery from lysed cells up to 4 days is probably related to intracellular multiplication, declining numbers after this time probably relate to a declining cell population excacerbated by some degree of cell loss during washing rather than a decline in intracellular bacteria.

5.2.3 Viable counts of infected PK15 cells lysed by Nonidet P40 (NP-40) after the elimination of extracellular bacteria

a) Introduction

The homogenisation of washed infected PK15 cells (5.2.2) did not disrupt all the cells.

In this experiment, an initial attempt was made to assess the ability of NP-40 to lyse all the cells in a culture and to examine whether or not CSM grew in the presence of NP-40. Subsequently infected cells were washed free of bacteria, then lysed with NP-40 and the number of organisms determined by the surface viable count method.

b) Assessment of lysis of PK15 cells by Nonidet P40

i) Preparation of NP-40 stock solution

A stock solution of NP-40 (5%) was prepared in PBS and sterilised by autoclaving at 10 lb/sq.in for 15 minutes. This solution was stored at room temperature until required, when the relevant dilution was prepared in PBS as listed in the details for the appropriate experiment.

ii) Lysis of PK15 cells by NP-40

After removing the supernatant medium, 3-day-old confluent monolayers of PK15 cells grown in 4 oz medical flats were overlaid with 10 ml of either 0.5, 1, or 2% NP-40 solution and incubated at 37°C for 20 to 30 minutes. The lytic effect of NP-40 on the cell sheet was determined every 5 minutes using a light microscope.

c) Results

Lysis of the cell sheets began soon after exposure to NP-40 and the process appeared to be complete within 5 minutes in 2% , 10 minutes in 1% and 20 minutes in 0.5% solution with the formation of a gel-like clot in the supernatant fluid. Vigorous mixing with a pipette disintegrated the gel into fine strands of cell debris.

d) Growth of CSM in the presence of NP-40

Two dry CBA plates were spread uniformly with 0.1 ml of 0.5, 1 or 2% NP 40 and held at room temperature for 15 minutes. These plates were then overlaid with 0.1 ml of a suspension of a 24-hour culture of CSM in PBS, and incubated at 37°C in a hydrogen microaerophilic atmosphere. Plates were examined for the presence of bacterial growth after 24 and 48 hours. Control CBA plates overlaid with 0.1 ml of sterile PBS and inoculated with 0.1 ml of CSM suspension were incubated and examined similarly.

e) Results

This experiment attempted to simulate the release of viable CSM from cells plated on bacteriological media in the presence of NP-40. Assessment of the growth present on the surface of CBS plates treated with NP 40 and on control plates showed no inhibitory effect due to the NP 40.

Additional tests confirmed that NP-40 was not bactericidal to CSM at these concentrations, subcultures of CSM were readily obtained from the plates for up to 7 days of incubation in the presence of NP-40. The colonial morphology of CSM grown in the presence of NP-40 was similar to that obtained on untreated CBA plates.

This result confirms the report of Rajasekhar (1981) that treatment of CSM grown in culture with NP-40 does not affect the viability of these organisms. This property of NP-40 provides a useful means of lysing infected cell cultures without affecting the viability of this organism.

f) Production of cell lysis by NP-40 and viable counts of the lysate

Overnight cultures of PK15 cells in 4 oz medical flats were each overlaid with 10 ml of a suspension of a 24 hour culture of CSM in MEM and incubated at 37°C. Every day an infected monolayer was vigorously washed with warm PBS as previously described in 5.2.1. The washed infected cells were lysed by adding 10 ml of 1% NP 40 to the monolayer. After 10 minutes at 37°C the lysate was homogenised by vigorous mixing with a sterile pipette. The number of bacteria in the lysate was determined by the viable count method.

g) Results

Table 19: Viable counts of CSM from infected PK15 cells lysed by NP-40 after the elimination of extracellular bacteria

Time after infection in days	Last rinse of infected monolayer	Lysate of infected monolayer
1	NR	2.78*
2	NR	3.40
3	NR	3.85
4	NR	4.88
5	NR	4.54
6	NR	3.65
7	NR	2.30
8	NR	1.86
9	NR	NR
10	NR	NR

* = Count of CSM as $\log_{10}$ organisms per ml (corrected to two decimal places.

NR = No organisms recovered

Infecting dose = 10.76 $\log_{10}$ organisms per ml.

There was an increase in the number of CSM recovered from lysed infected PK15 cells in the first 4 days of incubation and thereafter a decrease until bacteria could not be isolated after 10 days (Table 19).

h) Comments

Although all infected cells were not lysed when a homogeniser was employed (5.2.2), the recovery of CSM (Table 18) was similar from homogenised cells and from cells lysed by NP-40 (Table 19).

5.2.4 Total viable count of 'unwashed' infected cells lysed with Nonidet P40

a) Introduction

It will be recalled that vigorous washing of MDBK cells infected with CSM detached the cells from the glass and the method did not eliminate bacteria from the supernatant of infected Int 407 cells (5.2.1).

In view of these observations and the difficulty of differentiating intracellular from cell-associated extracellular CSM the following experiment was undertaken to determine the "total yield" of viable bacteria irrespective of their situation either within or on the surface of the infected cells. Such information would be of value in interpreting the quantitative data provided by the viable counts of the supernatant fluids of infected cells.

b) Materials and Methods

The essential difference between this experiment and that described for infected PK15 cells was that in the latter case recovery of bacteria was assessed from lysed infected cells after elimination of extracellular bacteria. In this present experiment assessment of growth was made from the "total yield" of both intra and extracellular bacteria.

Confluent monolayers of MDBK or Int 407 cells grown overnight in 4 oz medical flats were overlaid with 5 ml of a suspension of a 24 hour culture of CSM in prewarmed (37°C) MEM and incubated at 37°C.

Infected monolayers of each cell type were lysed during each of the first 5 days' and on the 7th, 10th, 14th, 17th and 21st day post infection, by the addition of 5 ml of 1% NP-40 added directly to the supernatant tissue culture fluids. The NP-40 treated cultures were incubated at 37°C for 20 minutes and then the lysates vigorously homogenised with a sterile pipette. The cell homogenate were used to assay "total viable" CSM by the surface viable count technique. The number of organisms in the supernatant fluids of these infected cells were also determined by surface viable counts before the addition of NP-40.

c) Results

CSM was recovered from the supernatant fluid of infected MDBK cells at 21 days after infection whereas bacteria were only isolated from infected Int 407 cells up to and including the 17th day after infection (Table 20), and not thereafter. Both MDBK and Int 407 cells showed increases in the

Table 20: Total viable count of "unwashed" infected MDBK and Int 407 cells lysed by Nonidet P40 and recovery of CSM from the supernatant fluids

Time after infection in days	Infected MDBK cells		Infected Int 407 cells	
	Supernatant fluid	"Total" cell lysate and supernatant fluid	Supernatant fluid	"Total" cell lysate and supernatant fluid
0	10.90*	10.90	10.90	10.90
1	6.50	6.78	7.40	7.65
2	5.95	6.88	7.60	7.90
3	5.88	6.90	7.18	7.95
4	5.78	6.95	6.60	7.98
5	5.60	6.98	6.18	7.48
7	5.18	7.03	5.40	6.40
10	5.08	6.18	4.88	5.60
14	4.70	5.78	4.18	4.70
17	4.40	5.19	3.30	3.50
21	4.18	4.78	NR	NR

* = Count of CSM as $\log_{10}$ organisms per ml (corrected to two decimal places).

NR = No organisms recovered

number of bacteria recovered after lysis of the cells compared with the supernatant recovery. The number of organisms in the lysate of infected MDBK cells increased daily until 7 days after which there was a progressive decrease till the end of this experiment.

Recovery from lysed infected Int 407 cells was similar but reached its maximum at 4 days, and thereafter decreased until no bacteria were isolated on the 21st day of infection.

d) Comments

The results of this experiment clearly indicate that multiplication of bacteria has occurred associated with the cells of the culture. It is interesting to note that the "total yield" of bacteria after lysis of infected cells was invariably higher than that from the supernatant fluids. In MDBK cells this indicates that the CSM isolated after lysis of cells were derived from intracellular multiplication since earlier observations (Chapter 4) showed that in this cell line no extracellular cell-associated bacteria are seen 24 hours post infection. However, in Int 407 cells there was an increase in the number of organisms in the lysate in the first 4 days of infection. It appears that this may be as a result of both intracellular and extracellular multiplication of CSM, since during this time bacteria are still attached to the cell surface of infected cells (Chapter 4).

In both cell lines the isolation of bacteria from infected cells is associated with the survival of infected cells and as a consequence there is a decrease in bacterial counts in the supernatant fluids and lysates towards the later part of the experiment.

5.3 Assessment of intracellular multiplication by immunofluorescence staining

5.3.1 Introduction

The recovery of viable bacteria after lysis of infected cells has indicated that intracellular multiplication of CSM takes place in PK15 and MDBK cells. The situation with Int 407 cells is less clear and organisms may proliferate on the surface or intracellularly in this time.

In an effort to resolve this point infection of these cell lines was examined by immunofluorescence staining using both formalin-fixed and acetone-fixed preparations.

5.3.2 Materials and Methods

Twenty four-hour-old cultures of PK15, MDBK or Int 407 cells grown as coverslip monolayers were infected with a 24 hour-old culture of CSM suspended in warm MEM as previously described in Chapter 2. Infected and uninfected coverslip preparations incubated at 37°C were collected daily for the first 5 days and on the 7th, 10th, 13th, 17th and 21st days. Coverslips were rinsed in several changes of PBS to remove unattached bacteria and then fixed for 10 minutes in ice cold acetone or for 30 minutes in 10% neutral formalin (Fraser, 1976). Immunofluorescence staining of these prefixed monolayers was carried out using rabbit anti-CSM serum (strain 253/72 - 'OH' antiserum) and FITC labelled sheep anti-rabbit immunoglobulin by the method described in Chapter 2.

5.3.3 Results

Examination of stained monolayers of PK15, MDBK and Int 407 cells fixed in acetone showed an increase in the number of brightly stained particles as time after infection progressed. Monolayers fixed in formalin failed to show the presence of any specific fluorescence on the cell membrane or within the cytoplasm of infected PK15 or MDBK cells from 24 hours onwards. Int 407 cells fixed in formalin showed bacteria attached to the cell surface at 24 hours after infection and thereafter.

Infected PK15 monolayers showed brightly stained coccoid and vibrioid forms within the cytoplasm after 24 hours after infection (Fig. 21). At 48 hours almost all the intracellular bacteria had lost their typical vibrioid morphology. The coccoid forms were present in the perinuclear cytoplasm and tended to aggregate to form large bright areas of fluorescence. There was no evidence of nuclear fluorescence. During days 3 - 5 after infection these coccoid forms greatly increased in number with large bright areas of fluorescence in the cytoplasm (Fig. 22). From the 6th - 9th day post-infection there was extensive cell sheet destruction and the remaining islets of cells continued to show intracytoplasmic fluorescence.

Examination of MDBK coverslip cultures 24 hours post infection showed fewer intracellular vibrioid and coccoid forms of the bacteria than PK15 cells (Fig. 23). After 5 days of infection there was an increase in the number of both forms of intracellular bacteria. The cell surface remained free of attached bacteria and the intracellular forms of CSM appeared to undergo rapid multiplication. Monolayers examined on 14th day post-infection showed that the cytoplasm of most cells contained enormous numbers of brightly stained particles some of which it could be

Fig. 21. PK15 cells 24 hours after infection with CSM showing intracytoplasmic brightly stained coccoid and vibrioid bacterial forms.
Acetone fixation, immunofluorescence staining with anti-CSM serum and sheep anti-rabbit (FITC) conjugate.

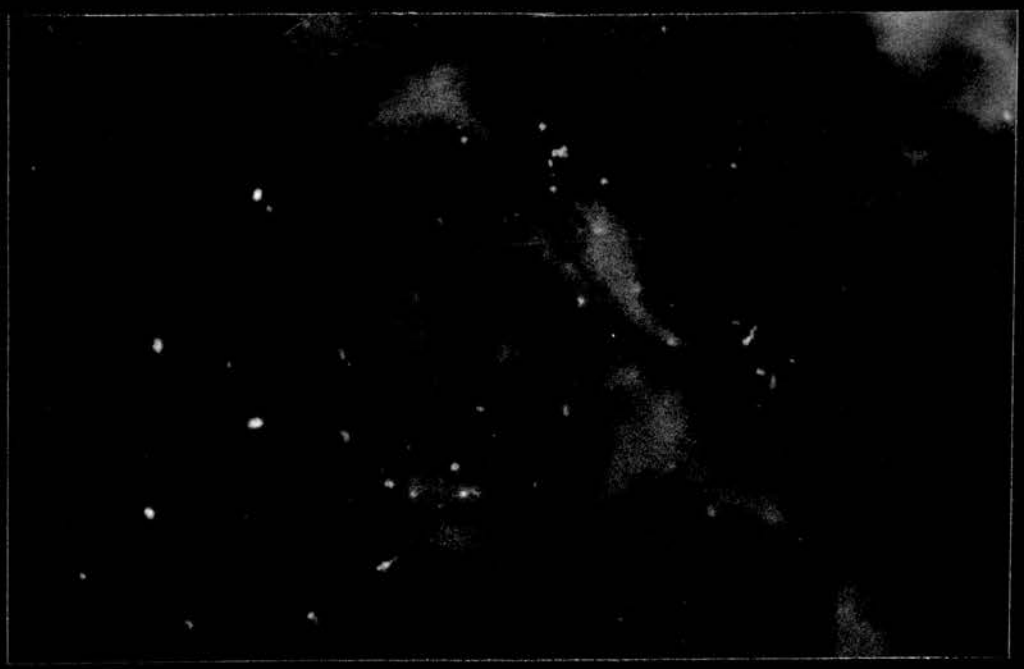
(x 284)

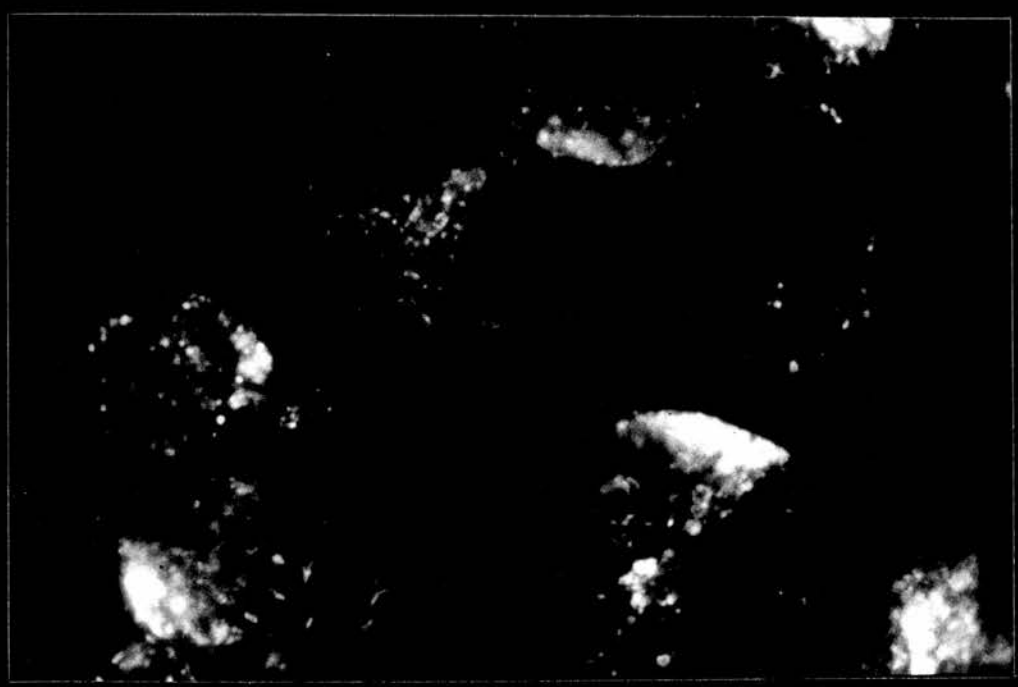
Fig. 22. PK15 cells 5 days after infection with CSM. Bacterial antigen is present as both coccoid and vibrioid forms. Note increased cellular antigen compared with that after 24 hours of infection (Fig. 21).
Acetone fixation, immunofluorescence staining with anti-CSM serum and sheep anti-rabbit (FITC) conjugate.

(x 284)

Fig. 23. MDBK cells 24 hours after infection with CSM. Only a few intracytoplasmic vibrioid and coccoid bacterial forms are present.
Acetone fixation, immunofluorescence staining with anti-CSM serum and sheep anti-rabbit (FITC) conjugate.
(x 180)

Fig. 24. MDBK cells 14 days after infection with CSM. Increased numbers of predominantly coccoid and occasional vibrioid bacterial forms are present.
Acetone fixation, immunofluorescence staining with anti-CSM serum and sheep anti-rabbit (FITC) conjugate.
(x 180)





appreciated were coccoid and some vibrioid (Fig. 24).

Acetone fixed Int 407 cells infected for 24 hours showed many coccoid and vibrioid forms of the bacteria with the latter form mainly apparently attached all over the cell surface (Fig. 25). After 7 days of incubation there was a decrease in the vibrioid forms and increase in the coccoid brightly stained particles. Examination of coverslip culture after 14 and 21 days showed a negligible number of vibrioid forms and the peri-nuclear area almost free of this form of bacteria. The cytoplasm appeared full of bright stained coccoid forms arranged around the nucleus (Fig. 26).

5.3.4 Comments

The inability of specific antiserum to penetrate formalin-fixed cells was confirmed in the present experiment where infected PK15 or MDBK cells invariably failed to show intracytoplasmic fluorescence despite the demonstration of enormous numbers of fluorescing intracytoplasmic organisms when cells were prefixed in acetone. These results confirm the original observations of Rajasekhar (1981).

Immunofluorescence staining of CSM-infected PK15, MDBK and Int 407 cell cultures showed that the bacteria remained on the surface of Int 407 cells longer than on MDBK or PK15 cells. There was a progressive increase in the number of fluorescing particles in all infected cell types which increased with time of incubation.

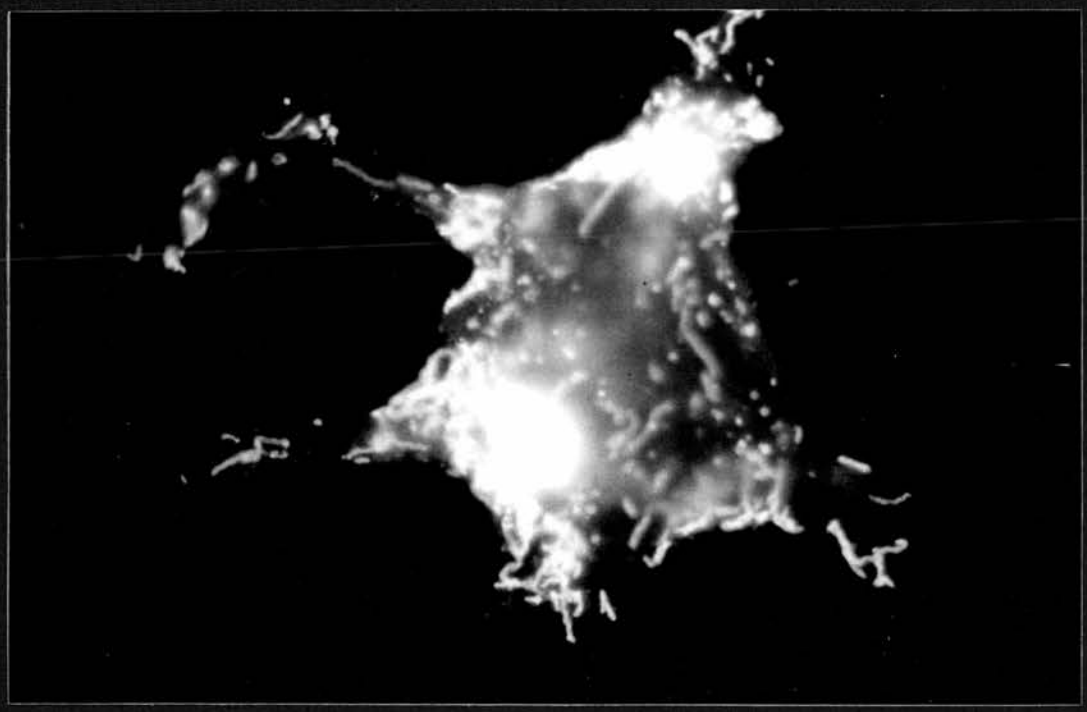
The change of intracellular bacteria from vibrioid to coccoid forms appears to be similar in PK15 and MDBK cells. After 24 hours of infection both bacterial forms were seen in the cells. At this stage of infection the cytoplasm contained large amounts of particulate material and estimation

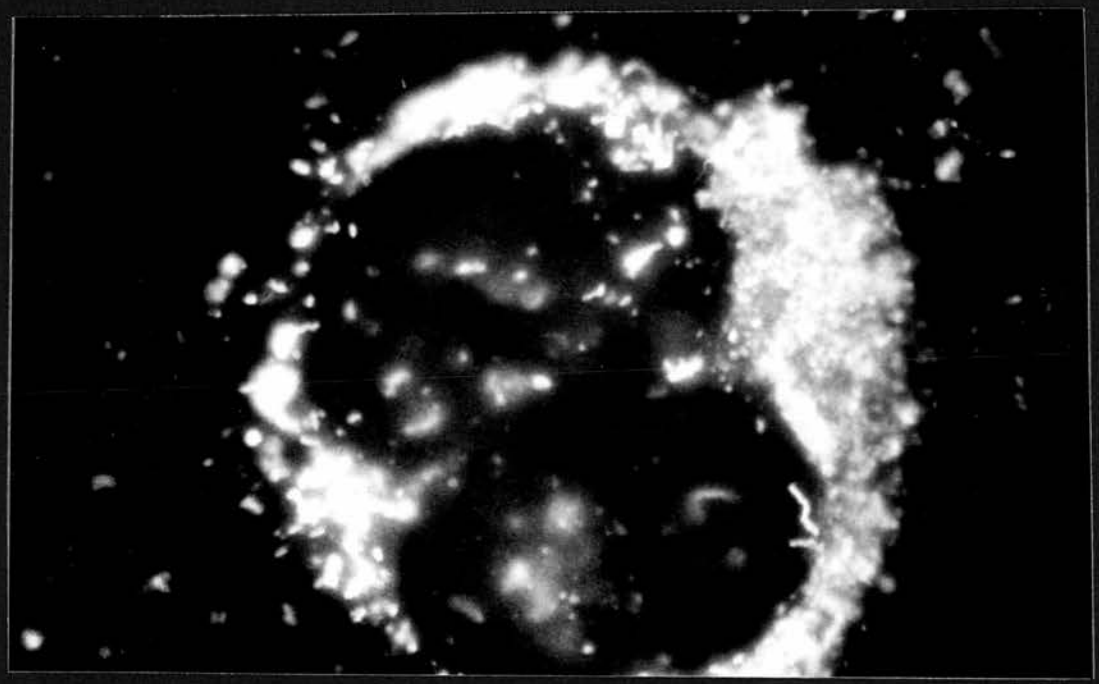
Fig. 25.

Int 407 cells 24 hours after infection with CSM.
Both coccoid and vibrioid bacterial forms are present.
Note the attachment of vibrioid forms all over the cell surface.
Acetone fixation, immunofluorescence staining with anti-CSM and sheep anti-rabbit (FITC) conjugate.
(x 284)

Fig. 26.

Int 407 cells 14 days after infection with CSM.
There has been a large increase in the numbers of intracytoplasmic coccoid forms and only a few vibrioid forms remain.
Acetone fixation, immunofluorescence staining with anti-CSM serum and sheep anti-rabbit (FITC) conjugate.
(x 284)





of the relative properties of the two forms was not exact, these reported differences may therefore not be significant. Towards the later stages of infection the fluorescing particles were predominantly coccoid forms.

In infected Int 407 cells the sequence of events somewhat differs from those seen in PK15 or MDBK cells. After 24 hours of infection formalin-fixed monolayers of 407 cells showed vibrioid forms while acetone-fixed monolayers demonstrated both vibrioid and coccoid forms. Therefore, the assumption is that the coccoid forms are intracellular. There was thereafter a progressive decrease in the number of extracellular coccoid forms. At about 21 days post-infection when bacteria were absent from the cell surface, all the intracellular bright stained particles were coccoid in shape.

5.4 Discussion

Earlier results (Chapter 4) showed that the peak periods of CSM attachment to PK15 and MDBK cells were 2 and 6 hours post-infection respectively, while that of Int 407 cells was 24 hours after infection. Rajasekhar (1981) showed that CSM did not survive in cell-free MEM under aerobic conditions. The marked drop in bacterial counts in the supernatant fluids of infected cells during the first 24 hours is probably a combined result of death of organisms in the supernatant fluid and attachment to and entry of CSM into cells.

After 24 hours of infection in PK15 cells organisms were not seen on the cell surface but were observed within the cell. There was a progressive increase in bacterial counts as well as fluorescing particles for the first 4 days of infection. Both vibrioid and coccoid

forms of bacterial antigen were demonstrated by immunofluorescence staining and the coccoid forms became dominant as the infection progressed; the number of intracellular viable bacteria in the culture decreased as the infected monolayer started to disintegrate and during the accumulation of intracellular coccal antigen.

After 24 hours of infection Int 407 cells showed vibrioid organisms attached to the cell surface and this population increased in number for the first few days before decreasing progressively and ended with an absence of attached extracellular bacteria after 21 days of infection. As the number of extracellular-vibroid forms decreased there was an increase in the number of intracellular coccoid forms. There was no increase in viable bacteria associated with the increase in intracellular fluorescing particles in the later stages of the experiment. This indicates that many of these particles were degraded CSM antigen.

In infected MDBK cells the sequence of events were similar to those in infected PK15 cells but the cells persisted and organisms were recovered from both the supernatant fluids and infected cell for at least 21 days. The early increase in viable bacteria suggests that this was possibly the period of maximal intracellular multiplication in MDBK cells.

These results have gone some way to defining the growth of CSM in cultures of PK15, MDBK and Int 407 cells. Evidence for intracellular bacterial multiplication was provided by viable counts from infected cell lysates and immunofluorescence staining showing cell associated bacteria. Growth studies detected cell-associated growth of bacteria but did not indicate that significant multiplication took place in supernatant fluids of infected cells. The progressive increase of intracellular particles in infected cells towards the later stages of the experiment were not reflected in the numbers of viable bacteria suggesting that much of the

increase was an increase in bacterial antigen and not viable bacteria. Several workers have relied on immunofluorescence staining to assay the intracellular growth of pathogenic bacteria (Horwitz and Silverstein, 1980; Daisy et al, 1981), the present study however clearly underlines a major limitation of this method.

CHAPTER 6.

Chapter 6

Infection of PK15 cells with tissue culture-derived CSM (CSM-Tc) and attempts to passage the bacteria in cells

Chapter 6

Infection of PK15 cells with tissue culture-derived CSM (CSM-Tc) and attempts to passage the bacteria in cells

6.1 General Introduction

Earlier observations showed that marked cytopathic changes were produced in PK15 cells exposed to a "standard" inoculum of CSM-c and that these changes were either associated with intracellular multiplication or possibly with the intracellular presence of non-viable CSM. This association between cell and bacteria may have effects on the bacteria that are isolated directly from infected cells. The effects of exposing cells in culture to bacteria directly isolated from infected cells have not been examined so it is not known whether tissue culture-derived organisms produce similar effects to those of cultured bacteria.

Most bacteria are not obligate parasites and grow on nutrient media when incubated under the right conditions of atmosphere and temperature. This feature, coupled with the difficulty of controlling the multiplication of bacteria in the extracellular fluid of cultured cells, explains why tissue culture cells are rarely used for the continuous propagation of bacteria. Viruses due to their obligate parasitic nature have regularly been continuously propagated and maintained in cell culture for a variety of laboratory purposes.

Having obtained evidence of bacterial intracellular multiplication by viable counts, immunofluorescence staining and other procedures, (Chapter 5) it was considered important to ascertain whether CSM derived from tissue culture infection (CSM-Tc) also had the ability to induce

cytopathic changes and to multiply in cells in a similar manner to CSM-c. Since these organisms do not multiply in the extracellular fluid of infected cells, attempts were made to passage CSM in PK15 cells.

6.2 The effects of exposing PK15 cells to CSM-Tc

In Chapter 3 it was shown that a "standard dose" of CSM-c induced a variety of cytopathic changes in PK15 cells. In this experiment bacteria obtained directly from lysed infected PK15 cells were used to infect cells in an attempt to ascertain whether CSM-Tc induced different cytopathic changes to bacteria derived from cell free culture.

6.2.1 Materials and Methods

PK15 cells were grown overnight in 4 oz medical flats. After discarding the supernatant fluid the monolayer was overlaid with 10 ml of a suspension of 24 hour-old CSM culture in warm MEM, and incubated at 37°C. After 4 days of incubation the supernatant fluid of the infected monolayer was discarded and the monolayer vigorously washed to eliminate extracellular bacteria as described previously in 5.2.1. The washed infected monolayer was lysed by adding 5 ml of 1% Nonidet P40 to the monolayer as described in 5.2.3. The lysate was washed twice in warm MEM by centrifugation at 4000 x g for 20 minutes and the fluid pellet resuspended in 5 ml MEM. One or 5 ml respectively of this suspension was used to infect 24 hour-old PK15 cells grown either on coverslips or in medical flats. The infected coverslips and medical flat cultures

were incubated at 37°C and everyday coverslips were removed for Giemsa and immunofluorescence staining while infected medical flat cultures were viewed by light microscopy for evidence of cytopathic changes. Each day the number of bacteria in the supernatant fluid was determined by the viable count method. Coverslip and medical flat cultures not infected with the lysate of infected cells were used as controls.

6.2.2 Results

Table 21: Infection of PK15 cells with CSM-Tc: cytopathic changes, intracellular parasitism and the recovery of CSM from supernatant fluids.

Time after infection in days	Cytopathic changes and intracellular parasitism		Bacterial count in supernatant fluid as $\log_{10}$ organisms per ml
0			4.78
1	0	(8)	2.90
2	0	ND	2.95
3	0	(4)	1.70
4	0	ND	NR
5	0	(2)	NR
6	0	ND	NR
7	0	(0)	NR
8	0	ND	NR
9	0	(0)	NR
10	0	ND	NR

NR = No organisms recovered

ND = Not examined

0 = No CPE

( ) = Numbers in brackets indicating the percentage of cells showing intracellular fluorescence.

The infecting dose of CSM derived from lysed infected cells was 4.78 $\log_{10}$ organisms per ml. Giemsa-stained preparation of PK15 cells exposed to the lysate of infected cells for 1 hour showed no bacterial attachment to the cells. Cytopathic changes were not detected in PK15 cells during the 10 days after exposure to infection with CSM-Tc, infected cells remained similar to uninfected PK15 cells in Giemsa-stained and unstained preparations (Table 21). At 24 hours after infection 8% of the cells showed small amounts of intracytoplasmic fluorescence (less than 10 fluorescing particles per cell) in acetone-fixed infected monolayers. As time after infection progressed, the percentage of cells showing fluorescing particles decreased until after 5 days no more fluorescence was demonstrated in infected cells. Bacteria were not recovered from the supernatant fluids of infected cells after 3 days.

6.2.3 Comments

Experiment 3.3 showed that CSM-c did not induce cytopathic changes in PK15 cells at an infecting dose of 5.26 $\log_{10}$ organisms per ml. The infecting dose of CSM-Tc used in this experiment was 4.78 $\log_{10}$ organisms per ml which also failed to induce any visible changes in PK15 cells. CSM-Tc therefore has behaved like CSM-c in that it failed to induce changes in PK15 cells at concentrations around 5 $\log_{10}$ organisms per ml.

In the same manner neither CSM-Tc nor CSM-c at low infection levels persisted in PK15 cells as revealed by immunofluorescence staining and bacterial recovery. It was not possible to assess the effect of exposure of cells to a dose similar to the CSM-c "standard dose" since

such a concentration of CSM-Tc could not be recovered from infected cells. The timing of the lysis of infected PK15 cells in this experiment was chosen to obtain the highest possible number of released bacteria.

6.3 Attempts to passage CSM-Tc in PK15 cells

6.3.1 Introduction

This experiment was carried out to explore further the possibility of continuously maintaining CSM in PK15 cells using either washed lysed infected PK15 cells or the supernatant fluids from infected cultures.

6.3.2 Materials and Methods

This is an extension of experiment 6.2 in which in addition a parallel experiment was performed where one-day-old PK15 cells were infected with the supernatant fluids containing CSM from infected PK15 cells.

i) Passage of CSM from lysed infected cells in PK15 cells

The first passage of CSM-Tc in PK15 cells (6.2) was performed as previously described in 6.2.1. After 2 days of incubation of PK15 cells infected with CSM-Tc (first passage), the monolayer was washed, lysed and used to infect 24-hour-old PK15 cells grown either on coverslips or in medical flats as previously described (6.2.1). The whole process was repeated for the third passage.

ii) Passage of CSM from superantant fluid of infected PK15 cells

After 2 days of the primary infection of PK15 cells with CSM, 1 ml or 5 ml of the supernatant fluids were withdrawn and used to infect uninfected 24-hour-old PK15 cells grown either on coverslips or in medical flats as appropriate. These cells were incubated at 37°C and every day coverslips were removed and stained by Giemsa and immunofluorescence. The number of bacteria in the supernatant fluids was determined daily by the surface viable count method. Supernatant fluids from cells infected with CSM-Tc that had been incubated for 2 days post infection were also used to infect 24-hour-old PK15 cells. This was also repeated in a further attempted bacterial sub-culture. Procedures were performed as described above for the first passage.

For clarity the initial CSM infection of PK15 cells is considered as the primary CSM infection.Tissue culture cells infected with second-day supernatant fluids containing CSM will be described as the first passage. Subsequent exposure of fresh PK15 cells to first passage supernatant fluid are therefore second and third passage as appropriate.

6.3.3 Results

Table 22a: Passage of CSM from lysed infected cells in PK15 cells: recovery of bacteria from supernatant fluids and intracellular parasitism of CSM

Time after infection in days	1st Passage		2nd Passage	
	Bacteria counts in supernatant fluid as $\log_{10}$ organisms per ml	Percentage of cells showing intracellular fluorescence	Bacterial counts in supernatant fluid as $\log_{10}$ organisms per ml	Percentage of cells showing intracellular fluorescence
0	4.78		2.95	
1	2.90	8	NR	0
2	2.95	ND	NR	0
3	1.70	4	ND	0
4	NR	ND	ND	ND
5	NR	2	ND	ND
6	NR	ND	ND	ND
7	NR	0	ND	ND
8	NR	ND	ND	ND

NR = No organisms recovered

ND = Not examined.

Table 22b: Passage of CSM from supernatant fluids of infected cells: recovery of bacteria from supernatant fluids and intracellular parasitism of CSM

Time after infection in days	1st Passage		2nd Passage		3rd Passage	
	Bacterial count in supernatant fluid as $\log_{10}$ organisms per ml	Percentage of cells showing fluorescence	Bacterial count in supernatant fluid as $\log_{10}$ organisms per ml	Percentage of cells showing fluorescence	Bacterial count in supernatant fluid	Percentage of cells showing fluorescence.
0	6.40		4.18		2.88	
1	4.10	12	2.70	6	NR	0
2	4.18	ND	2.88	ND	NR	0
3	3.54	8	1.70	4	NR	0
4	2.70	ND	NR	ND	ND	ND
5	1.88	6	NR	1	ND	ND
6	NR	ND	NR	ND	ND	ND
7	NR	3	NR	0	ND	ND
8	NR	ND	NR	ND	ND	ND
9	NR	0	NR	0	ND	ND
10	NR	ND	NR	ND	ND	ND
11	NR	0	NR	ND	ND	ND

NR = No organisms recovered

ND = Not examined.

The infecting doses from infected cell lysates for the first and second passages were 4.78 and 2.95 $\log_{10}$ organisms per ml respectively.

In PK15 cells infected with CSM derived from the lysate of infected cells, bacteria were recovered from the supernatant fluids during the first 3 days in the first passage. The number of intracellular fluorescing particles decreased as the days of infection increased. In the second passage bacteria were not recovered from the supernatant fluids and no fluorescing cell-associated particles could be demonstrated (Table 22a). The infecting doses from the supernatant of infected cells for the first, second and third passages were 6.40, 4.18 and 2.88 $\log_{10}$ organisms per ml respectively. PK15 cells infected with supernatant fluid of infected cells containing CSM yielded bacteria from the supernatant fluids during the first 5 and 3 days in the first and second passages respectively. Intracellular fluorescence decreased along with the recovery of the viable bacteria as time passed. During the third passage bacteria were neither recovered from the supernatant fluids nor detected intracellularly by immunofluorescence staining (Table 22b).

There was no evidence of cytopathic changes in cells infected with either CSM derived from the lysate or supernatant fluid of infected cells.

6.4 Discussion

Giemsa-stained preparations of PK15 cells infected with CSM derived from either the infected cell lysate or supernatant fluid did not show bacterial attachment after 1 hour of infection. In Chapter 4 it was shown that CSM-c started attaching to PK15 cells after 20 minutes of infection and reached a peak after 1 - 2 hours. It appears from this

result that there is a difference between CSM-Tc and CSM-c. One such difference may be bacterial motility, for subjective assessment appeared to show that bacteria from supernatant fluids of infected cells were less motile than 24 hour-old CSM culture suspended in MEM. This coupled with the low infecting dose may explain why bacteria attachment was not demonstrated in cells exposed to CSM-Tc 1 hour post-infection. Non-motile bacteria are unlikely to become motile in the supernatant fluid as this environment is unfavourable for bacterial survival; this effect would reduce still further, the bacteria capable of attaching to cells. The bacteria available to adhere to cells will therefore be considerably fewer than manifest by viable counts and this will in time have an effect on both the production of cytopathic changes and attempts to propagate the bacteria in cells.

Attempts to passage CSM derived from either the lysate or supernatant fluid of infected cells were not successful since there was a general decrease in the number of bacteria on each passage. It has been shown that the number of organisms in the inoculum is crucial to the production of cytopathic changes in PK15 cells and these results suggest that the same may apply to the propagation of the bacteria in cell culture. The inocula used in these experiments were 4.78 $\log_{10}$ and 6.40 $\log_{10}$ organisms per ml respectively and it has been previously demonstrated that a CSM inoculum of 5.26 $\log_{10}$ organisms per ml showed limited survival and little cytopathic effects in infected cells.

The evidence obtained so far suggests that irrespective of the source of origin of CSM, either culture or tissue culture, they behave alike in infected cells. Until a method is devised for recovering large numbers of CSM from tissue culture infection this limited comparison between the effects of these two sources of CSM could be misleading.

CHAPTER 7.

Chapter 7

A comparative study of infection of PK15 cells with cultured CSM (CSM-C) or CSM derived from adenomatous tissue (CSM-T)

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7.2 Development of cell culture infection techniques

7.2.1 Growth of CSM in the presence of antibiotics

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7.3 Development of techniques for the preparation of bacterial filtrates from PIA or PHE tissues

7.3.1 Introduction

7.3.2 Source of tissues from porcine proliferative enteropathies

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Chapter 7

A comparative study of infection of PK15 cells with cultured CSM (CSM-C) and CSM derived from adenomatous tissue (CSM-T)

7.1 General Introduction

Since the first demonstration of campylobacter-like profiles in the cytoplasm of adenomatous intestinal epithelium about a decade ago by Rowland and Lawson (1974), infection experiments employing affected tissues derived directly from affected animals have only been performed in experimental animals or embryonated hens' eggs (Lawson et al, 1979; Roberts et al, 1980a, 1980b; Lomax et al, 1982a; McCartney et al, 1984). Infection experiments using CSM or adenomatous tissue as inocula have either failed to reproduce the disease or have reproduced the condition inconsistently in experimental animals. The failure to reproduce the disease using CSM in gnotobiotic animals might be explained in a number of ways. Amongst those that should be considered are i) that cultured bacteria behave differently or have lost pathogenicity in comparison with tissue-derived organisms, ii) that CSM is not the intracellular organism, or iii) that lesions contain other bacteria along with CSM and that these other bacteria cannot be recovered under the cultured conditions that have been used. The porcine proliferative enteropathies are comparable pathogenically to a disease of hamster, transmissible ileal hyperplasia (TIH) or proliferative ileitis which involves an intracellular vibrio-like organism. The disease can be transmitted in experimental animals (Jacoby, 1978) and the intracellular agent grown in tissue culture (Jacoby and Johnson, 1981) using mucosal cell suspensions prepared from affected hamsters as inocula. Many attempts to isolate the intracytoplasmic organism in cell-free media

have been unsuccessful (Boothe and Cheville, 1967; Tomita and Jonas, 1968; Goldman et al, 1972; Wagner et al, 1973; Jacoby et al, 1975; Amend et al, 1976).

The work of Rajasekhar (1981) has helped define the behaviour of cultured CSM in tissue culture cells derived from a variety of animal species. If the lesions of PIA contain an intracellular bacteria other than CSM the evidence available suggests that it cannot be grown on conventional bacteriological media and may therefore require specialised growth factors or be cell-dependent. The most comparable groups of bacteria to the non-acid fast, Gram negative obligate intracellular bacteria are the Rickettsia and Chlamydia and the properties and techniques used in investigating these organisms may bear significance to the investigation of the possible unknown agent of PIA. Many workers have reported the infection of cell lines by such bacteria, for example, in 1975 Burgdorfer et al described the infection and the rapid spread of Rickettsia rickettsii in tissue culture monolayers and similar observations were made by Wisseman et al (1976) in chicken embryo fibroblast cell culture. In addition to the rapid spread of infection, Wisseman and colleagues found that a substantial number of organisms accumulated extracellularly rather than in the cytoplasm while the presence of compact intranuclear masses in some of the cells suggested that R. rickettsii was capable of penetrating the nucleus from the cytoplasm. Later, working with chicken embryo fibroblasts, Silverman and Wisseman (1979) described rapid changes in the morphology of the cells infected with R. rickettsii. In electron micrographs of infected cells several progressive host cell lesions were observed. These included widespread dilation of the rough endoplasmic reticulum and outer nuclear envelope, associated with accumulation of electron dense material within the cisternae of the intracellular membranes. The rickettsiae were found free

in the host cell cytoplasm, or within the invaginations of the nuclear envelope but not within the cisternae formed by the swollen endoplasmic reticulum. As a result of intracisternal swelling and fusion of intracellular membranes during the later stages of the infection cycle, the majority of rickettsiae were surrounded by host cytoplasm bound by host-derived internal membranes and appeared to persist in this state until cell lysis occurred. In contrast infection and intracellular growth of Rickettsia prowazekii in chicken embryo fibroblasts appeared to produce less dramatic effects (Silverman et al, 1980). Cytopathic changes were not apparent until late in the intranuclear growth cycle when cells heavily laden with large numbers of rickettsial bodies began to rupture. The only recognisable changes in heavily infected cells before lysis occurred was condensation of the interstitial matrix of some mitochondria and the apparent dissociation of ribosomes from the rough-surfaced endoplasmic reticulum. Byrne (1978) worked with mouse fibroblasts (L-cells) and Chlamydia psittaci and suggested that attachment and ingestion of large numbers of chlamydiae might cause simultaneous injury both to the plasma membrane itself and to the metabolism of the cell. Support for this view was provided by scanning electron micrographs of monolayers of L-cells inoculated with high multiplicities of Chlamydia which showed direct damage to the host cells with rounding up and detachment from the glass (Moulder et al, 1976; Byrne, 1978). In summary, a variety of obligate intracellular Rickettsia and Chlamydia have been cultured successfully in tissue culture cells, these can be readily observed microscopically and frequently produce severe cytopathic changes in the infected cells. Successful infection of cell cultures with tissue-derived campylobacter-like organisms might resolve some of the problems involved in the aetiology of PIA, in particular it might provide evidence for the presence of any as yet uncultivated agent or agents capable of

multiplying in tissue culture cells.

This chapter describes attempts made to infect a line of porcine kidney cells (PK15) with bacteria derived directly from diseased adenomatous tissue (CSM-T). Comparisons were made during the course of the work with cells exposed to cultured CSM (CSM-C). Efforts were also made to passage cell infection which had been initiated with bacteria derived from adenomatous tissue.

7.2 Development of cell culture infection techniques

In an attempt to obtain inocula from adenomatous tissue that contained cell derived campylobacters free from other contaminating bacteria a combination of techniques was employed namely:- i) incorporation of antibiotics in the diluent used to prepare suspensions from adenomatous tissue ii) trypsinisation of adenomatous tissue to disaggregate cells iii) homogenisation of the trypsinate to lyse and to separate organisms from cells and iv) filtration of the homogenate to remove contamination organisms.

The antibiotics used were those employed extensively in the routine isolation of CSM from clinical and experimental adenomatous material. These have been shown to have little or no effect on the viable counts of CSM derived either from clinical material or culture but inhibit a range of other organisms (Lawson, personal communication).

Different techniques have been used by several workers to disintegrate cells in order to release intracellular contents. These include mechanical disruption in tissue homogenisers, ultrasonic disruption of cells or cell lysis by chemicals such as Nonidet P-40. Ultrasonic disruption was not used to separate intracellular organisms from cells because of the possible damaging effects on the viability of intracellular organisms. The experiments

described in Chapter 5 showed that Nonidet P-40 lysed CSM-infected tissue culture cells without any effects on the intracellular organisms; it is not known whether this chemical would affect tissue-derived organisms and so to minimise possible harmful effects of chemicals this method was not used in the preparation of tissue lysates.

Filtration is employed to sterilise fluids or solutions that are thermolabile. Membrane filters made from a variety of synthetic materials have been widely used in the separation of a number of types of bacteria from larger organisms in contaminated material (Lawson et al, 1979; Gebhart et al, 1983). This procedure has been utilised particularly in the separation of the spirochaetes and campylobacters which due to their small diameter filters more readily than other organisms and which before the development of selective media were frequently overgrown by more robust bacteria. Membrane filters have considerable advantages over other types of filter as the variation in pore size are less, allowing more accurate separation of the bacteria. Membrane filters of various pore sizes were therefore used in the filtration of homogenised adenomatous tissue.

In previous cell infection experiments cells were grown and infected with cultured CSM in 6" x 5/8" Pyrex test tubes or 4 oz medical flats. As the volume of filtered fluid derived from infected mucosa might be relatively small and to avoid undue dilution of the inocula cells were grown and infected with filtrate in 2 ml-screw-capped vials and 50 ml NUNC disposable tissue culture bottles.

Initial experiments were undertaken to assess the effect of the procedures proposed for tissue-extracted bacteria on CSM infection of PK15 cells.

7.2.1 Growth of CSM in the presence of antibiotics

7.2.1 (a) Introduction

Antibiotics or antibacterial drugs were selected on the basis of those unlikely to affect the growth of CSM and with a high minimum inhibitory concentration against the majority of strains of the organism (Lawson and Rowland, 1984). The agents used might be expected to act on a range of intestinal contaminating bacteria which may not be the same or have the same properties in all cases of the disease. Clearly the choice of antibacterials is restricted as many agents are active against CSM.

The ability of CSM to grow in three different dilutions of a combination of antibacterial drugs was examined in this experiment.

7.2.1 (b) Materials and Methods

i) Preparation of antibacterial solutions

Nalidixic and stock

This was prepared by dissolving.05 gm of Nalidixic acid (Sigma N4382) in 50 ml distilled water to give 1 mg/ml stock solution. The minimum amount of 10 M NaoH was added to this suspension until the Nalidixic acid dissolved to give a clear solution.

Rifampicin stock

This was prepared by dissolving 0.1 gm Rifampicin (Sigma R3501) in 20 ml methyl alcohol and thereafter diluting 2 ml of this alcoholic Rifampicin solution with 8 ml distilled water to give a 1 mg/ml stock solution.

Trimethoprim or Novobiocin stock

Each solution was prepared by dissolving either .05 gm of Trimethoprim (Sigma T7883) or .05 gm of Novobiocin (Sigma N1628) separately in 50 ml of distilled water to give 1 mg/ml stock of each antibiotic.

Table 23 Antibacterial drugs used during the preparation of suspensions from adenomatous tissue

Antibacterial drug	Concentration of stock solution in mg/ml	Antibacterial supplement		
		x 1	x 2	x 3
Trimethoprim (T 7883)	1[a]	5[c]	10	15
Rifampicin (R 3501)	1[a]	5	10	15
Novobiocin (N 1628)	1[a]	5	10	15
Nalidixic acid (N 4382)	1[a]	40	80	120
Fungizone	5[b]	25	50	75

a Sigma Chemical Co. Ltd., Dorset, England

b E.R. Squibb and Sons, Linc., Princeton, New Jersey, U.S.A.

c Working concentration in μg/ml.

Fungizone stock

This was prepared by dissolving 0.25 gm of Fungizone (E.R. Squibb) in 50 ml of distilled water to give a stock solution of 5 mg/ml.

All stock solutions were stored frozen at -30°C, when required they were thawed and diluted in MEM to the required working dilutions as listed in Table 23.

ii) Treatment of CSM with antibiotic solutions

To 50 ml of each antibiotic supplement (x 1, x 2, x 3) in a universal container was added 5 ml of a 24 hour CSM culture suspended in MEM. The bacterial suspension added to MEM without antibiotics was used as a control. Cultures were incubated at 37°C and the viability of organisms in the fluids assessed by spreading 0.1 ml on duplicate CBA plates at two-hourly intervals for the first 12 hours and at 24 hours post-inoculation.

In a parallel experiment duplicate CBA plates were spread with 0.1 ml of antibiotic supplement x 1, x 2 or x 3 in MEM. Control plates were spread with 0.1 ml MEM. These plates were held at room temperature for 10 minutes before each was overlaid with 0.1 ml of 24 hour CSM culture suspended in MEM. All plates were incubated in a microaerophilic atmosphere at 37°C and examined after 48 hours.

7.2.1 (c) Results

The number of bacterial colonies recovered on CBA plates from fluids that contained antibiotic supplement x 1 or 2 were similar to the controls. Fewer bacteria were recovered from the x 3 supplement media from 4 h and thereafter in comparison with the other supplements and the control. There were no differences in the size or morphology of bacterial colonies recovered from the control or antibiotic-included media. Although there were a few colonies of about half the diameter of typical CSM colonies these were recovered from all the media in similar numbers. Subsequent subculture after

Table 24 Effect of antibiotics on the growth of CSM: recovery of bacteria from cell-free MEM

Time after inoculation in hours	Quantitative recovery of bacteria from cell-free fluids			
	Without antibiotics	With antibiotics		
	Control	x 1	x 2	x 3
0	++++[a]	++++	++++	++++
2	+++	+++	+++	+++
4	+++	+++	+++	++
6	++	++	++	+
8	++	++	++	+
10	+	+	+	-
12	+	+	-	-
24	-	-	-	-

- = No bacterial growth

a = Quantitative assessment of bacterial growth

++++ Confluent growth of CSM, decreasing to +++, ++ and + where only a few bacterial colonies were recovered.

48 hours from all plates to CBA plates yielded viable organisms.

There was no recovery of CSM from the fluid culture with antibiotic supplement x 3 eight hours after inoculation, while the control and x 1 showed no bacterial growth after 12 hours. Bacteria were recovered from fluids containing antibiotic supplement x 2 up till 10 hours but not after this period (Table 24).

Similar numbers of bacterial colonies grew on CBA plates containing antibiotic supplement x 1 or x 2 and the control plates. Plates that contained supplement x 3 showed an apparent reduction in the number of colonies.

7.2.1 (d) Comments

These results indicated that exposure to antibiotic supplement x 2 did not affect the subsequent growth of CSM and higher levels appeared only to accelerate the normal lethal effect of media exposed to oxygen at atmospheric tension. Cell culture media containing such antibiotics might be expected to reduce or eliminate contaminating bacteria in inocula derived from adenomatous intestine without seriously affecting the viable counts of CSM. Such inhibition of contaminants might make possible the infection of cell culture with field derived inocula. The few micro colonies of CSM recovered from both antibiotic-containing and antibiotic-free media was not a result of exposure to antibiotics but possibly represents organisms damaged by oxygen exposure.

7.2.2. Growth of cells in the presence of antibiotic supplement (x 2)

7.2.2. (a) Introduction

In this experiment antibiotic supplement (x 2) was assessed for its effect on the growth of PK15 cells.

7.2.2 (b) Materials and Methods

A confluent monolayer of PK15 cells was trypsinised, centrifuged at 200 x g for 1 minute and the pellet resuspended in growth medium with antibiotic supplement (x 2). Cells were grown on coverslips as well as in medical flats and refed with maintenance medium containing antibiotic supplement at 3 days intervals throughout the experiment. Every day for 10 days coverslips were removed and stained by Giemsa's method. Cells grown in medical flats and stained coverslips were examined by light microscopy for cellular abnormalities. Control cells grown and refed in appropriate medium without antibiotics were processed similarly.

7.2.2 (c) Results

Neither Giemsa-stained coverslips nor unstained monolayers of PK15 cells grown in the presence of antibiotics differed in growth or morphology from the control cells at any stage in the experiment.

7.2.3 Effect of antibiotic supplement (x2) on the attachment of CSM to PK15 cells and on the recovery of bacteria from the supernatant fluids

7.2.3 (a) Introduction

It has been shown that the antibiotic supplement (x 2) had no effects on the growth of either PK15 cells or CSM. It was considered necessary to assess whether the presence of these antibiotics in the culture medium affected either the attachment of CSM to PK15 cells or the recovery of bacteria from the supernatant fluids of infected cells.

7.2.3 (b) Materials and Methods

PK15 cells were grown on coverslips in the presence of antibiotic supplement (x 2) for 24 hours. The supernatant fluid was discarded and overlaid with 1 ml of 24 hour old CSM culture suspended in MEM with antibiotic supplement (x 2).

Infected coverslips were incubated at 37°C, removed hourly for the first 4 hours, rinsed in warm PBS and stained by Giemsa's method. Thereafter everyday for 10 days the effect of the antibiotics on the recovery of CSM was assessed by spreading 0.1 ml of the supernatant fluids from infected cells on duplicate CBA plates. These plates were incubated in a microaerophilic atmosphere for 48 hours. Cells grown on coverslips in MEM without antibiotics were infected with 24 hour-old CSM culture suspended in MEM, processed as above and used as controls.

7.2.3 (c) Results

i) Attachment of CSM to cells

Cells grown and infected with CSM in the presence of antibiotics showed a pattern and degree of bacterial attachment that was similar to the control cells for the 4 hours of the experiment.

ii) Recovery of CSM from supernatant fluids of infected cells

No differences could be detected in the number of bacteria recovered from the antibiotic-containing and antibiotic-free supernatant fluids at each stage throughout the period of this experiment (Table 25). In both series CSM could be recovered up to and including day 9 of the experiment.

Similar cytopathic changes were observed in both cells grown and infected with CSM in the presence or absence of antibiotics. These changes were similar to those described in Chapter 3 for PK15 cells infected with

Table 25 Recovery of CSM from supernatant fluids of infected PK15 cells in the presence of antibiotic supplement (x 2)

Time after infection in days	Quantitative recovery of bacteria from cell supernatant fluids	
	Containing antibiotics	Without antibiotics
0	++++	++++
1	+++	+++
2	+++	+++
3	+++	+++
4	+++	+++
5	++	++
6	++	++
7	++	++
8	+	+
9	+	+
10	-	-

- = No bacterial growth

a = Quantitative assessment of bacterial growth

++++ Confluent growth of CSM from 0.1 ml supernatant, decreasing to +++, ++ and + where o nly a few bacterial colonies were recovered from 0.1 ml.

CSM and observed in preparations stained by Giemsa's method.

7.2.3 (d) Comments

The addition of antibiotics to the growth medium for PK15 cells and to the medium in which CSM was suspended did not have any effects on the attachment of the bacteria to the cells. These antibiotics therefore did not in any way modify or alter either the receptor sites on PK15 cells or the bacterial characters involved in attachment to the cells. The numbers and duration of bacterial recovery both appeared to indicate that the pattern of cell infection was very similar whether it took place in the presence of antibiotic supplement (x 2) or not.

7.3 Development of techniques for the preparation of bacterial filtrates from PIA or PHE tissues

7.3.1 Introduction

Crude homogenates of adenomatous intestinal tissue contains along with campylobacters a variety of other intestinal organisms. The preparation of an inoculum for cell infection from affected mucosa containing only intracellular derived campylobacters could involve a number of techniques. These might include the use of antibiotics in the culture medium at strategic points in the preparation of the inocula, differential filtration or centrifugation, lysis of infected cells to free intracellular bacteria and serial dilution to free the predominant intracellular organism from other bacteria.

In these experiments attempts were made to develop a satisfactory method for the preparation of an inoculum containing campylobacter-like organisms from adenomatous tissue which could be subsequently used for infection

experiments with PK15 cells.

7.3.2 Source of tissues from porcine proliferative enteropathies

The sources, and reference numbers of the tissues from proliferative enteropathy cases used in these investigations are listed in Table 26.

Intestinal material was obtained immediately after death from killed animals or fatal clinical disease. The diseased intestine was processed either by separating the epithelium from the muscle coat followed by homogenisation freezing and storage at -70°C or the intact intestine was ligated and frozen at -70°C. In the former case aliquots were prepared by partially thawing the bulk and dispensing portions of tissue which were rapidly returned to -70°C. In the latter case the intestine was partially thawed, the mucosa removed, homogenised, dispensed as small aliquots and returned to -70°C. Homogenisation was carried out in each case using an MSE blender at 13,000 rpm in 30 ml containers.

In all cases the lesions were confirmed histologically as being typical of PIA or PHE and intracellular bacteria were demonstrated by one or more of a number of techniques namely: modified Ziehl-Neelsen, Silver staining or electron microscopy. This work was carried out by either A.C. Rowland, Dr E. McCartney or Dr G.H.K. Lawson.

Frozen rather than fresh adenomatous intestinal material was used in these experiments for a variety of reasons. It is not always possible to predict the availability of suitable clinical material and there are practical advantages in ensuring that tissues used for infection are pathologically typical of the proliferative enteropathies before experiments are undertaken. The use of frozen tissue also provides the opportunity to use materials from the same source for repeated experiments, an essential feature for the development of techniques for satisfactory cell infection. It should be

remembered however that results with fresh adenomatous material may be different from frozen mucosa and that the freezing process might adversely affect the viability of some organisms in the tissues.

7.3.3 Determination of the concentration and time of action of trypsin that will disaggregate the cells of adenomatous tissues

7.3.3 (a) Introduction

Adenomatous intestinal material although homogenised before storage in the frozen state still contained aggregates of intact cells on thawing. Separation and lysis of infected cells is likely to be necessary for the release of the maximum number of intracellular bacteria. Trypsinisation of the tissue prior to secondary homogenisation might promote dispersal and lysis of the cells and the release of intracellular campylobacters.

Since it is not clear how sensitive tissue-derived bacteria are to trypsin or to manipulation under atmospheric oxygen tensions, this experiment assessed the maximum concentration of trypsin that will disperse cells from adenomatous tissue in the shortest possible time.

7.3.3 (b) Materials and Methods

i) Preparation of working dilutions of trypsin

Stock solution of 1% (w/v) trypsin in PBS frozen at $-30^{\circ}C$ was thawed and from it dilutions containing 0.25, 0.50 and 0.75% trypsin were prepared in PBS.

Table 26 Sources, microbiology and pathology references of adenomatous intestinal tissues used in the investigations

Source	Died or Killed	Microbiology reference	Pathology reference	Pathological description
MLC Stirling	K	1080/76	BB 96/77	PHE
MLC Selby	D	1269/76	BB 108/76	PHE
Gorgie Slaughterhouse Edinburgh	K	363/78	BB 51/78	PIA
Gorgie Slaughterhouse Edinburgh	K	175/78	BB 22/78	PIA

ii) Weighing and centrifugation of adenomatous intestinal tissues

An aliquot of PIA tissue (363/78) was thawed, weighed and suspended in a known volume of MEM containing antibiotic supplement (x 2). From this suspension a $^{1}/_{100}$ dilution of the tissue was made in MEM containing antibiotics, 10 ml amounts were then dispensed into each of 4 centrifuge tubes and the remaining volume stored at -30°C. The tubes with contents were centrifuged at 4000 x g for 20 minutes. The supernatant fluids were discarded, the pellets resuspended each in 10 ml PBS, centrifugation repeated and the pellets retained.

iii) Trypsinisation of tissue pellets

The pellet from each tube (ii) was resuspended separately in 5 ml of either 0.25, 0.50, 0.75 or 1% trypsin in a 15 ml bijoux bottle. A magnet was introduced into each bottle, the bottles with contents were placed on a Chemlab magnetic stirrer (Chemlab, England) and stirred at mark 4 for 1 hour at 37°C. Every 10 minutes a few drops from each bijoux were placed on a glass slide, air-fixed and stained by the Brucella differential technique as described previously in Chapter 2. At the same time 0.1 ml of each trypsinate was spread on duplicate dried (CBA) plates and incubated microaerophilically at 37°C for 48 hours.

7.3.3 (c) Results

After 40 minutes of the experiment suspensions of adenomatous tissues exposed to 0.75 or 1% trypsin were uniformly turbid with no visible discrete particles or sediment. Microscopy showed large numbers of bright red stained campylobacter-like organisms many contained within apparently intact cells, although the presence of free bacteria indicated that some of the cells had been lysed at some stage in the process. Aggregates of intestinal cells

were not seen. The fluid of tissue suspensions exposed to 0.25% trypsin showed very little turbidity during the experiment with most of the tissues remaining in floccules or clumps. There was disaggregation although incomplete of cells in tissue exposed to 0.50% trypsin. Brucella differential stained smears of tissues exposed to either 0.25% or 0.50% trypsin showed clusters of bright red campylobacters located within cells. All suspensions exposed to trypsin treatment yielded CSM to the end of the treatment period and although not quantified numerically these appeared to be at a maximum after 40 minutes with 0.75% or 1% trypsin. Although there was progressive disaggregation of the tissues at all trypsin concentrations with time, only 0.75% and 1% gave homogenous solutions after 40 minutes.

7.3.3 (d) Comments

The exposure of adenomatous tissues to either 0.75% or 1% trypsin showed identical results in both disaggregation of cells and recovery of CSM from the trypsinate. Since the aim of the experiment was to achieve maximum cell disaggregation with the lowest trypsin concentration all subsequent trypsinisation of adenomatous tissues were performed with 0.75% trypsin.

7.3.4 Homogenisation of trypsinised cells to release intracellular bacteria

7.3.4 (a) Introduction

Brucella differential stained preparations of smears of trypsinised adenomatous material showed that the bright red campylobacters were still in clusters and lying apparently within the cells. It seems that although the cells had been disaggregated by the trypsin many cells were still intact and the majority of the bacteria had not been released from the host cell

cytoplasm.

This experiment was undertaken to evaluate homogenisation subsequent to trypsinisation of mucosal tissue as a method for the disruption of cells and release of organisms.

7.3.4 (b) Materials and Methods

Aliquots of adenomatous tissues were weighed, diluted, washed by centrifugation and trypsinised as described, then 5 ml MEM containing antibiotics was added to 5 ml of the trypsinate to stop the action of trypsin. The mixture was next homogenised at 13000 rpm on an overhead blender (MSE, Cawley, England) for 30 seconds in a 30 ml container cooled by ice.

Drops of the homogenate were air-fixed on a glass slide and stained by the Brucella differential technique. The number of organisms in the homogenate was determined by spreading 0.1 ml on duplicate CBA plates. The plates were incubated microaerophilically at 37°C for 48 hours.

7.3.4 (c) Results

Brucella differential-stained smears of the homogenate showed that the bright red campylobacters had been separated from the cells and were no longer in clusters but were present simply in the film. Assessment of bacterial recovery also showed that more viable organisms were obtained from the homogenate than from the trypsinate.

7.3.4 (d) Comments

The homogenisation of trypsin treated adenomatous intestinal cells resulted in cell lysis and the release of organisms. Bacterial recovery of CSM appeared to be enhanced by trypsinisation and homogenisation, such a

result might be expected as the bacteria of intracellular micro-colonies are likely to form single colonies on solid media.

7.3.5 Filtration of the homogenate through membrane filters of various pore sizes

7.3.5 (a) Introduction

The aim of filtration was to remove the bacteria that contaminate adenomatous tissue and to obtain the highest possible numbers of CSM in the final filtrate. Small pore size membrane filters have only a limited capacity to filter useful volumes of fluid heavily loaded with large diameter particles. It is essential therefore in order to obtain satisfactory filtration to remove the bulk of the large particles material before the critical filtration is carried out. This first step can be performed in a number of ways but most usefully by prefiltration with large pore size membrane filters or glass fibre pore filters.

7.3.5 (b) Materials and Methods

The membrane filters used in this experiment are listed in Table 27. Filtration was by positive pressure through 8.0, 0.8, 0.65 or 0.45 μm APD filters employing sterile disposable plastic syringes and Millipore swinnex holders sterilised by autoclaving at 10 lb per sq. inch for 10 minutes. Filtration through the non-autoclavable 0.60, and 0.50 μm APD filters used negative pressure and an all-glass Millipore filter apparatus (Ref. xx10025 00).

Table 27 Pore sizes and diameter of membrane filters used in the investigation

Filter type	Average pore diameter in μm	Diameter of filter in mm
Millipore filter* (SCWP 04700)	8.0	47
Millipore filter (AAWP 02500)	0.8	25
Millipore filter (DAWP 02500)	0.65	25
Millipore Polyric filter (BDWP 02500)	0.60	25
Millipore Celotate filter (EHWP 02500)	0.50	25
Millipore filter (HAWP 02500)	0.45	25

* Millipore U.K. Ltd., Millipore House, Middlesex, U.K.

i) Filtration of homogenate

Diluted, trypsinised and homogenised preparations of intestinal tissue (363/78) in 10 ml volumes were successively passed through 8.0, 0.8, 0.65, 0.60, 0.50 and 0.45 μm APD Millipore membrane filters. After filtration through each membrane 10^{-3} and 10^{-4} dilutions of the filtrate in MEM containing antibiotics were prepared. Dried CBA and RNBGT plates were then inoculated with 0.1 ml of each dilution and incubated under aerobic, anaerobic or microaerophilic conditions at 37°C for 48 hours.

ii) Preliminary characterisation of the isolates

Isolates from the filtrates were characterised using the following tests and utilising the OSM type culture (253/72) as a positive control.

Test for catalase was performed as recommended by Cowan (1974). Motility was sought by viewing a drop of the suspension of each isolate suspended in PBS under a light microscope as described previously (4.3.2). Slide agglutination was carried out by emulsifying surface growth of each isolate in a drop of PBS on a slide, to this was added a drop of anti-CSM serum (253/72) and the mixture rocked for 1 minute at room temperature before viewing. Strains were tested for their ability to grow under aerobic conditions by inoculating duplicate CBA plates with each colony type and then incubating the plates at 37°C for 48 hours.

iii) Scanning electron microscopy of filtrates or CSM-C

A few drops of an overnight culture of CSM suspended in warm MEM and each filtrate from adenomatous intestine filtered through 0.8, 0.6 and 0.45 μm membranes prepared as in (i) were fixed, stained as previously described in Chapter 2 and viewed with a Philips 505 scanning electron microscope at 30 KV.

7.3.5 (c) Results

i) Recovery of bacteria at various stages of filtration

The predominant bacterial type recovered on CBA plates from the homogenate or filtrates was type 3 which was evident in higher dilutions of material and only on plates incubated under microaerophilic (Table 28a) or anaerobic (Table 28c) conditions. Type 2 organisms were the least in number while type 1 were fewer than type 3 organisms. The incubation of the homogenate or filtrates on CBA under anaerobic conditions decreased the number of colonies of each type of organisms recovered in comparison to microaerophilic incubation. Only type 3 organisms were recovered on RNBGT plates from the homogenate or filtrates and the bacterial counts were lower than on CBA plates when each was incubated under microaerophilic or anaerobic conditions (Tables 28a, 28c). Only type 1 organisms were recovered on CBA plates incubated aerobically (Table 28b). Type 2 organisms were recovered on CBA but not on RNBGT plates from the homogenate up to 0.65μm filtrate when incubated under microaerophilic or anaerobic conditions.

The three types of bacteria passed through 8.0 and 0.8 μm filters, type 1 was held back by 0.65 μm filters as evidenced by scanning electron microscopy (SEM) of the filtrates and viable counts on CBA incubated under microaerophilic conditions (Table 28a). Only type 3 organisms grew on culture after filtration through 0.6 μm filters and SEM of this filtrate demonstrated only one bacterial type similar to CSM-C. Bacteria were neither recovered from filtrates through 0.50 nor 0.45 μm filters.

Brucella differential-stained smears of the homogenate up to 0.60 μm filtrate showed a decrease in the numbers of single bright red-stained campylobacters. Organisms were not seen in smears of 0.50 or 0.45 μm filtrates.

Table 28a Recovery of bacteria at various stages of filtration of homogenate of PIA tissue (363/78) after incubation under microaerophilic conditions for 48 hours.

Material plated	Bacterial count as $\log_{10}$ organisms per ml						Number of campylobacters seen in BDS[b]
	Colony type 1*		Colony type 2*		Colony type 3*		
	CBA	RNBGT	CBA	RNBGT	CBA	RNBGT	
Homogenate	5.46	NR	3.43	NR	5.78	4.93	++[c]
8 μm[a]	5.40	NR	3.40	NR	5.68	4.90	++
0.8 μm	3.00	NR	2.70	NR	4.85	4.60	++
0.65 μm	NR	NR	2.30	NR	4.60	4.34	+
0.60 μm	NR	NR	NR	NR	4.48	4.11	+
0.50 μm	NR	NR	NR	NR	NR	NR	-
0.45 μm	NR	NR	NR	NR	NR	NR	-

* Isolated colony types

a Successive filtration through membrane of given pore sizes

b Filtrate stained by Brucella differential stain, red stained campylobacters recorded

c Number of campylobacters seen - = None, + to ++ = increasing number

NR No organisms recovered

Tabl 28b Recovery of bacteria at various stages of filtration of homogenate of PIA tissue (363/78) after incubation under aerobic conditions for 48 hours

Material plated	Bacterial count as $\log_{10}$ organisms per ml					
	Colony type 1*		Colony type 2*		Colony type 3*	
	CBA	RNBGT	CBA	RNBGT	CBA	RNBGT
Homogenate	4.28	NR	NR	NR	NR	NR
8 μm[a]	4.20	NR	NR	NR	NR	NR
0.8 μm	2.18	NR	NR	NR	NR	NR
0.65 μm	NR	NR	NR	NR	NR	NR
0.60 μm	NR	NR	NR	NR	NR	NR
0.50 μm	NR	NR	NR	NR	NR	NR
0.45 μm	NR	NR	NR	NR	NR	NR

* Isolated colony types

a Successive filtration through membrane of given pore size

NR No organisms recovered

Table 28c Recovery of bacteria at various stages of filtration of homogenate of PIA tissue (363/78) after incubated under anaerobic conditi ons for 48 hours

Material plated	Bacterial count as $\log_{10}$ organisms per ml					
	Colony type 1*		Colony type 2*		Colony type 3*	
	CBA	RNBGT	CBA	RNBGT	CBA	RNBGT
Homogenate	4.20	NR	3.18	NR	4.60	4.28
8 μm[a]	4.04	NR	3.14	NR	4.45	4.20
0.8 μm	2.26	NR	2.62	NR	4.28	4.08
0.65 μm	NR	NR	2.18	NR	4.18	3.98
0.60 μm	NR	NR	NR	NR	3.95	3.80
0.50 μm	NR	NR	NR	NR	NR	NR
0.45 μm	NR	NR	NR	NR	NR	NR

* Isolated colony types

a Successive filtration through membrane of given pore sizes

NR No organisms recovered

Scanning electron microscopy of the homogenate, 8 or 0.8μm filtrate confirmed the presence of three bacterial types in each preparation. One bacterial type which was predominant was similar to CSM (Strain 253/72), these were curved rods with an irregular ridged surface. The second type were shorter thick rods with a smooth, straight margin, while the third were curved rods which differed from CSM (253/72) by being apparently thinner and having a smoother surface.

ii) Preliminary characterisation of isolates from filtrates

Colony type 1 on dried CBA plates after 48 hours of incubation averaged 3-3.5 mm in diameter and were creamy, circular and raised with a smooth surface. Type 2 colonies which did not exceed 2.00 mm in diameter were beige, convex and circular. Type 3 colonies were not more than 1.5 mm in diameter after 48 hours incubation and were circular, raised with a flat and shiny yellowish-grey surface.

All three bacterial isolates were motile, Gram negative rods (Table 28d). Types 2 and 3 were curved rods which did not grow aerobically and only type 2 was catalase positive. Positive slide agglutination with anti-CSM (253/72) serum was shown only by type 3 bacteria which confirmed their identity as CSM. Further repeated examination of aliquots of PIA 363/78 revealed the irregular presence of small numbers of C. coli/jejuni or on occasion C. hyointestinalis. Retrospectively, it is not certain of the exact identity of the strain with which this work was carried out, it will therefore be referred to as Campylobacter sp. (Lawson, personal communication).

Table 28d Preliminary characterisation of isolated from PIA tissue (363/78)

Test	Colony type			CSM-C
	1	2	3	
Gram stain	-	-	-	-
Bacterial form	R	CR	CR	CR
Catalase	-	+	-	-
Growth in oxygen	+	-	-	-
Motility	+	+	+	+
Slide agglutination with anti CSM (253/72) serum	-	-	+	+

R = Rods

CR = Curved rods

\+ = Positive

\- = Negative

7.3.5 (d) Comments

The results show that type 3 isolate has characteristics of CSM-C and scanning electron microscopy of 0.6μm filtrate showed organisms similar to cultured CSM. Such filtrates contained organisms that stain in the same way as the intracellular organisms and CSM can be isolated from the filtrate. Therefore 0.6 μm filtrates were used as a source of intracellular organisms and will be referred to in subsequent experiments as tissue-derived campylobacters which may or may not be synonymous with tissue-derived CSM (CSM-T).

7.4 Exposure of PK15 cells to each bacterial type isolated from PIA tissue

7.4.1 Introduction

In the last experiment it was shown that after filtration three types of bacteria could be isolated from the homogenised PIA tissue (363/78). This experiment was undertaken to examine the effect of pure cultures of each isolate on PK15 cells.

7.4.2 Materials and Methods

i) Growth of cells on coverslips in 2 ml screw-capped vials

The growth and infection of PK15 cells was performed in 2 ml screw-capped vials to reduce the possible effect of atmospheric oxygen tensions on tissue-derived bacteria. A confluent monolayer of PK15 cells was trypsinised, centrifuged at 200 x g for 1 minute, and the pellet resuspended in antibiotic-free MEM. The cell pellet was dispersed and dispensed in 1 ml amounts into

vials containing coverslips and the cells grown for 24 hours at 37°C.

ii) Growth of bacterial isolates

The three bacterial types isolated from the homogenate of adenomatous tissue were each subcultured twice on CBA plates to obtain pure cultures. Each culture was then grown on a CBA slope in a microaerophilic atmosphere at 37°C for 24 hours and resuspended in warm antibiotic-free MEM as previously described in Chapter 2.

iii) Infection of PK15 cells

The growth medium of the 24 hour-old PK15 cells was removed by pipetting and 1 ml of each bacterial suspension separately used to infect a cell monolayer. These vials and contents were incubated at 37°C for up to 10 days. As controls, cell monolayers were uninfected or infected with 1 ml of 24-hour-old CSM-C suspended in MEM. After 1 hour of infection, and thereafter daily coverslips were removed from each of the infected and uninfected vials, rinsed thoroughly in warm PBS, fixed in methanol and stained by Giemsa's method.

7.4.3 Results

Giemsa-stained coverslips of PK15 cells infected with type 3 isolates showed the same pattern and intensity of attachment as cells infected with cultured CSM. Types 1 and 2 did not show any attachment to cells.

After 24 hours the supernatant fluids of cells infected with type 1 or 2 turned yellow and had a foul smell while those of uninfected cells or cells infected with either type 3 of CSM-C remained reddish-purple. By the 2nd day cells infected with either type 1 or 2 had been totally detached

from the coverslips and were degenerate. Gram-stained preparations of supernatant fluids of these infected cells showed that the fluids contained one bacterial type in each case, and were not contaminated by any other organisms. Viable counts revealed that the number of organisms in the fluids had increased almost threefold during the two days of infection. Cells infected with either type 3 or CSM-C showed similar cytopathic changes as described in Chapter 3.

7.4.4 Comments

This experiment has shown that twoof the three types of bacteria isolated from PIA tissue were capable of multiplication in the extracellular fluid of infected cells. This made it impossible to study the cytopathic changes produced by either type 1 or 2 isolate in PK15 cells. The failure of attachment to PK15 cells and the rapid cytotoxic effects associated with extracellular growth would seem to suggest that neither type 1 or 2 organisms had the properties likely to be associated with persistent intracellular infections.

7.5 A comparative study of infection of PK15 cells with cultured CSM (CSM-C) or CSM derived from adenomatous tissue (CSM-T)

7.5.1 Introduction

It has been shown (Chapter 3) that a "standard dose" of cultured CSM consistently induced particular cytopathic changes in PK15 cells. In experiment 7.3.5 it was demonstrated that although filtrates of homogenised PIA tissues through 8, 0.8 and 0.65 μm membranes contained CSM, such

preparations were not suitable for the study of the effect of intracellular bacteria on cells because of the presence of contaminating types 1 and 2 organisms. These organisms multiplied in the extracellular fluid and caused rapid cell death and termination of the experiment.

In this experiment PK15 cell monolayers were exposed to either an 0.6 μm filtrate which was known to contain CSM or cultured CSM to compare the subsequent cytopathic changes.

7.5.2 Materials and Methods

Filtrates used for the infection of cells were prepared from aliquots of two PIA tissues 363/78 and 175/78 (Table 25).

i) Growth of PK15 cells

A week-old confluent monolayer of PK15 cells was detached from the glass with STV, centrifuged at 200 x g for 1 minute, the supernatant fluid discarded and the pellet resuspended in MEM containing antibiotics. Cells were dispensed at a concentration of 1 x 10^5 cells per ml in 1 or 10 ml amounts into 2 ml screw-capped vials or 50 ml-Nunc bottles as appropriate and grown as coverslip monolayers at 37°C for 24 hours.

ii) Preparation of CSM-C inoculum

Cultured CSM was grown for 24 hours on a CBA slope in a microaerophilic atmosphere at 37°C as previously described in Chapter 2. The surface growth was removed by gentle washing with 10 ml of warm antibiotic-free MEM.

iii) Preparation of filtrate containing CSM derived from PIA tissue

a) Weighing and centrifugation of PIA tissue

The weight of an aliquot of PIA tissue was determined by a similar method to that described in 7.3.3. Then a 10^{-2} dilution of the tissue was made in MEM containing antibiotics. Ten ml of the suspension was removed, centrifuged at 4000 x g for 20 minutes, the supernatant fluid discarded, replaced by 10 ml PBS and centrifugation repeated. After discarding the supernatant fluid the pellet was trypsinised.

b) Exposure of the adenomatous pellet to 0.75% trypsin

Trypsinisation of the pellet was performed by a similar method to that described in 7.3.3. Briefly, the pellet was resuspended in 5.0 ml 0.75% trypsin with a magnet in a bijoux bottle. The bottle with its contents was placed on a Chemlab magnetic stirrer and stirred at mark 4 for 40 minutes at 37°C.

c) Homogenisation of the trypsinate

Homogenisation of the trypsinised tissue was performed in a 30 ml container of an MSE overhead blender at 13000 rpm for 30 seconds as described previously in 7.3.4.

d) Filtration of the homogenate

The homogenate was passed successively through membrane filters of 8.0, 0.80 and 0.60 μm A.P.D. by a similar method to that described in 7.3.5. The final filtrate 0.60 μm was used for the infection of PK15 cell monolayers. Brucella differential stained smears of filtrates were examined by light microscopy.

iv) Infection of cells with cultured CSM or filtrate containing CSM

Appropriate monolayers of PK15 cells grown for 24 hours were exposed to either 1 or 10 ml of 24-hour-old CSM culture suspended in warm antibiotic-free MEM or, 1 or 10 ml of the final filtrate and incubated at 37°C for 11 days.

Infected coverslip monolayers were removed after 1 hour and thereafter daily from each group. These were processed for Giemsa and immunofluorescence staining, transmission and scanning electron microscopy as described in Chapter 2. Uninfected coverslips were removed, processed as above and used as controls. The numbers of organisms in the supernatant fluids of infected cells were also determined daily by the surface viable count method (2.5.4) and the plates incubated at 37°C in a microaerophilic atmosphere for 48 hours.

7.5.3 Results

Surface viable counts of the filtrates from each PIA material (363/78, 175/78) on CBA showed that they contained one bacterial type, which also grew on RNBGT agar. Filtrate from 363/78 contained 4.70 $\log_{10}$ organisms per ml while 3.48 $\log_{10}$ organisms per ml were recovered from 175/78. Brucella differential-stained preparations of each filtrate showed bright red campylobacters in the smears.

i) One hour post infection

Giemsa and immunofluorescence staining, transmission and scanning electron microscopy of cells exposed to CSM-C showed the same characteristic pattern of attachment previously shown for PK15 cells and described in Chapters 3 and 4 (Figs.28,29). Bacteria could not be observed on the surface of

cells exposed to CSM-T using any of the four techniques (Fig. 27). There was no evidence of any cytopathic effects in cells infected with CSM from any source.

ii) 24 hour post infection

Bacteria were not recovered from the supernatant fluids of cells infected with CSM-T and there was the expected marked decrease in the number of organisms in the supernatant fluid of CSM-C-infected cells (Table 29). Organisms were not observed on the cell surfaces of cells infected with CSM derived from either source on examination by scanning electron microscopy. Cells infected with CSM-C or CSM-T, fixed in acetone and stained with anti-CSM serum showed intracellular stained vibrioid and coccoid forms. The majority of cells exposed to CSM-C showed intracellular antigen whilst only a few cells proved positive in those exposed to filtrates from 363/78 or 175/78 (Table 29). There were no visible cytopathic changes in Giemsa-stained preparations of cells infected with either cultured or tissue derived bacteria.

iii) Two days post infection

The cytopathic changes induced by CSM-C in this stage in the experiment were similar to those already described in Chapter 3 for PK15 cells infected with cultured organisms. Giemsa-stained preparations of cells infected by CSM-T showed no visible changes in the cells. Organisms were not recovered from the supernatant fluids of infected cells but there was intracellular bacteria parasitism evidenced by 12 and 15% respectively of cells showing bright stained fluorescing particles.

Cells with enlarged nuclei and cells starting to fuse were evident in

Fig. 27. Scanning electron micrograph of PK15 cells 1 hour after infection with PIA filtrate containing CSM. No bacteria are attached to the cell surface.

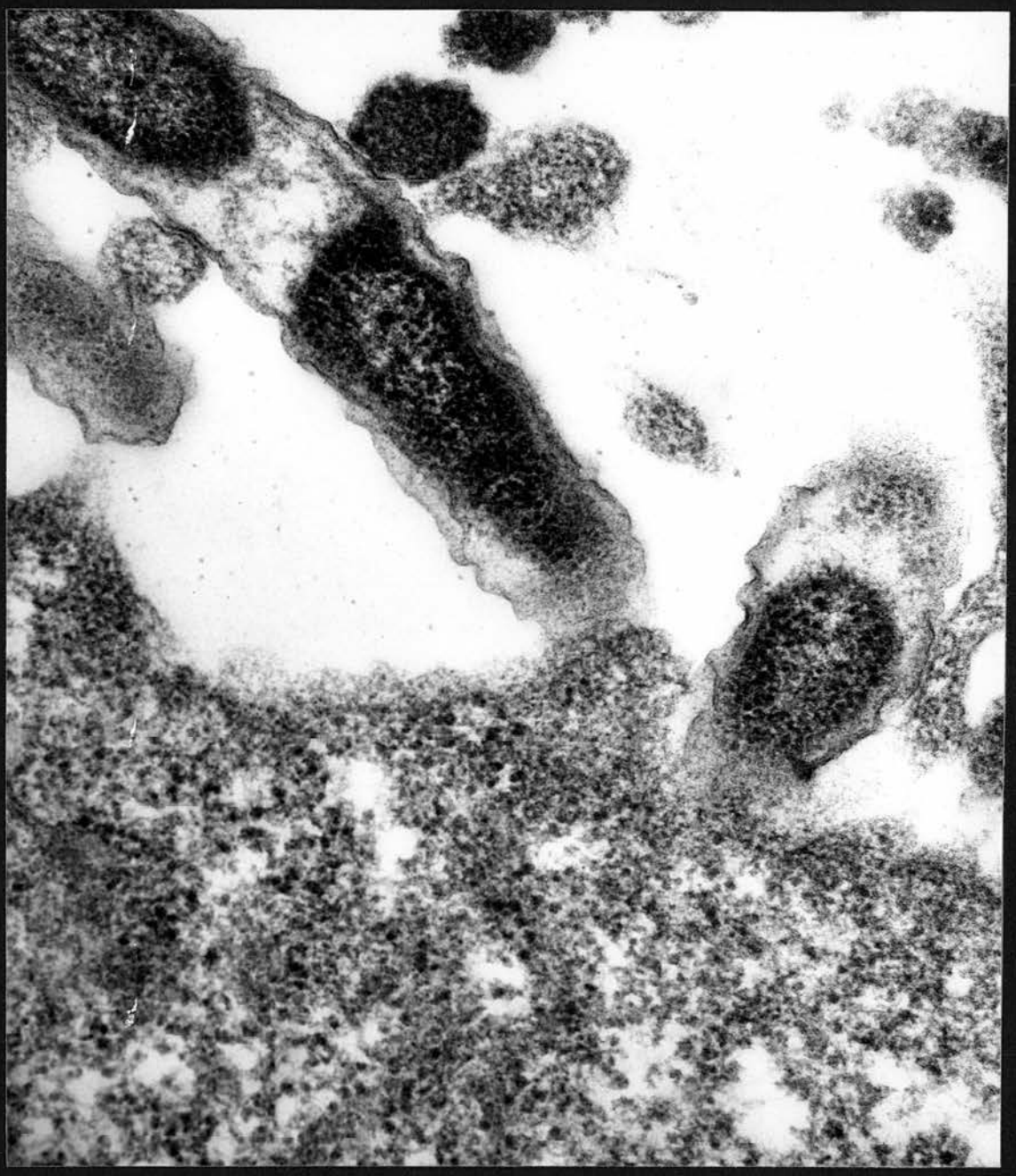
(x 6120)

Fig. 28. Scanning electron micrograph of PK15 cells 1 hour after infection with cultured CSM (CSM-C). Bacteria are attached to the cell surface in the normal manner.

(x 12040)

Fig. 29. Transmission electron micrograph of PK15 cells 1 hour after infection with CSM-C. Bacteria are intimately attached to the periphery of the cell.

(x 89750)



Giemsa-stained monolayers of CSM-C infected cells. Bacteria were recovered from the supernatant fluids and 95% of the cells showed intracellular fluorescing particles with anti-CSM serum (Table 29).

Bacteria were not seen on the surfaces of cells infected with bacteria from either source during this period of infection. Immunofluorescence stained preparations demonstrated intracellular fluorescing particles in cells infected with CSM from either source. Transmission electron micrographs of CSM-T infected cells showed intracellular bacterial structures which were curved in forms but had irregular, star-like outline lying freely in the cell cytoplasm (Fig. 30). Cells infected with CSM-C demonstrated organisms lying freely or surrounded by membranes in the cell cytoplasm.

iv) Three to eight days post infection

Organisms were recovered from the supernatant fluids of cells infected with CSM-T from the 3rd day after infection and the numbers increased daily till the 5th day after which there was a progressive decrease in the number of organisms until the fluids no longer yielded bacteria on the 11th day. There was a comparable daily increase in the number of cells showing intracellular parasitism of bacteria during this period of the experiment (Table 29). The supernatant fluid from cells infected with CSM-C showed a progressive decrease in the number of organisms and these cells demonstrated similar numbers of intracellular organisms to those observed in cells infected with CSM-T.

Unstained and Giemsa-stained preparations of cells infected with CSM-C or CSM-T showed extensive destruction of the monolayer and many cells were abnormal (Figs. 31, 32) compared with control cells (Fig. 33). Immunofluorescence stained preparations of cells infected with bacteria from either source

Fig. 30. Transmission electron micrograph of PK15 cells 3 days after infection with PIA filtrate containing CSM. Irregularly-shaped intracellular bacteria (arrowed) are present in the cytoplasm of the cell.

(x 19250)

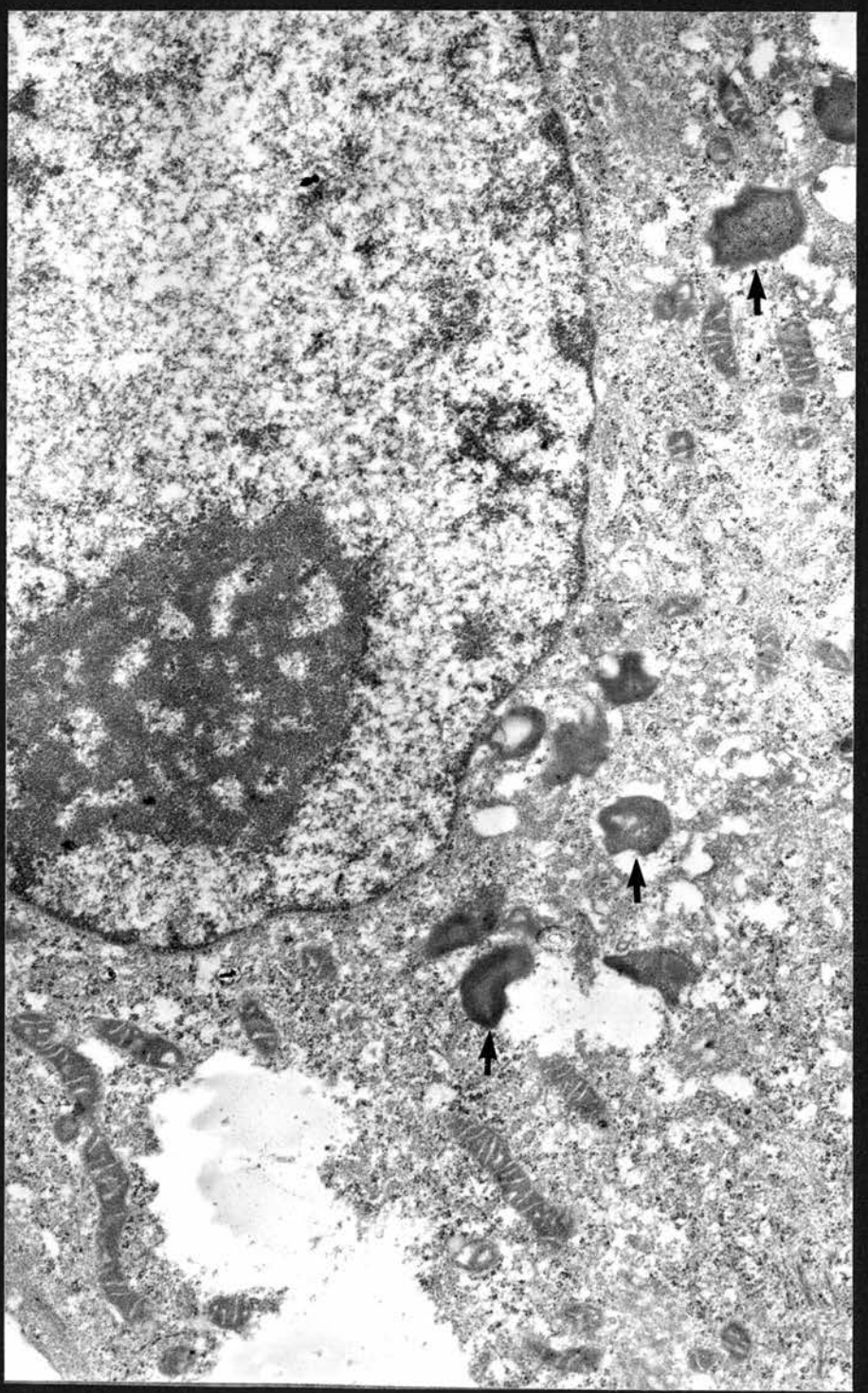


Fig. 31. Unstained monolayer of PK15 cells 5 days after infection with PIA filtrate containing CSM. Extensive cell loss and numerous abnormal cells can be seen.

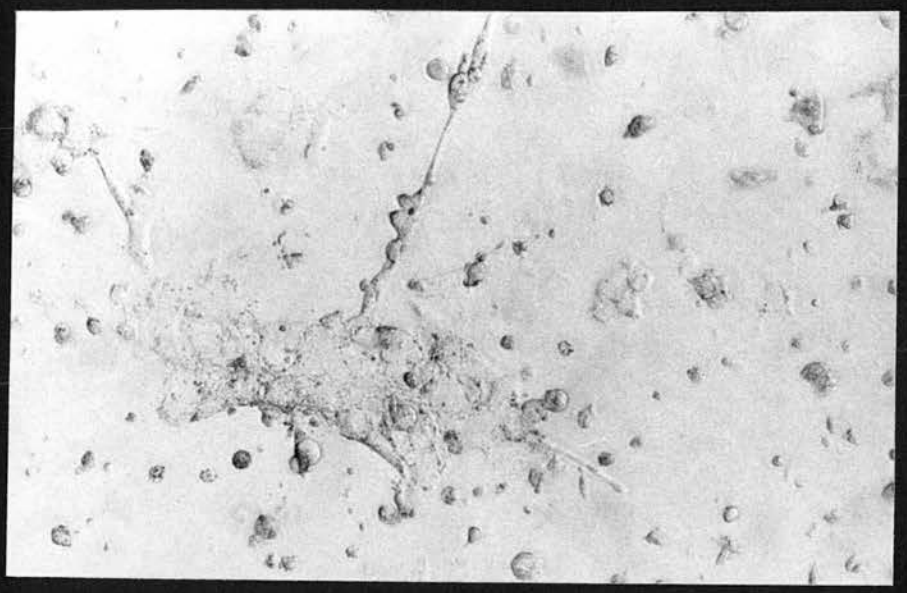
(x 45)

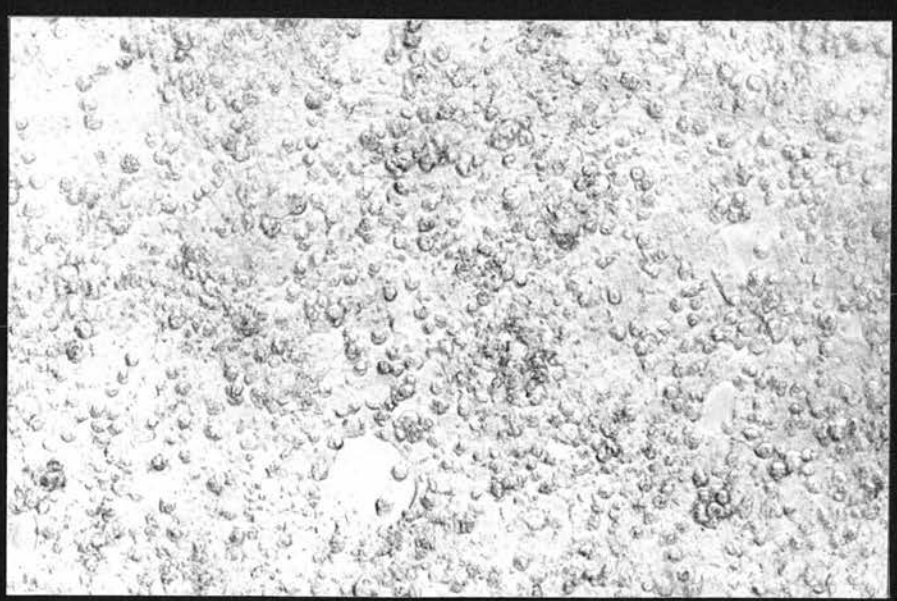
Fig. 32. Unstained monolayer of PK15 cells 5 days after infection with CSM-C. Abnormal cells with swollen cytoplasm can be seen.

(x 45)

Fig. 33. Unstained, uninfected 6 days-old PK15 cells showing a confluent monolayer.

(x 45)





showed increased number of intracellular fluorescing particles. Transmission electron micrographs of CSM-C infected cells demonstrated organisms in phagosomes surrounded by membranes and some lying freely in the cytoplasm. Bacteria at various stages of destruction in the phagocytic vacuoles were also shown in these preparations.
In CSM-T infected cells the increase in number of intracellular organisms was marked (Fig. 34) and this was further confirmed by immunoflourescence staining of cells infected for the same period. The intracytoplasmic filaments which are thought to have a cytoskeletal function were obviously visible during the later part of the infection in cells exposed to CSM-T but not in CSM-C infected cells. There was an increase in the number of these filaments from the 5th day till total destruction of the cell sheet (Fig.34).

v) Nine to eleven days post infection

In cells infected with cultured CSM very few organisms were recovered from the supernatant fluids 9 or 10 days post infection and bacteria were not recovered after 11 days (Table 29). Bacteria were not isolated from the supernatants of cells infected with tissue-derived filtrates after 10 or 11 days of infection (Table 29). Patches of infected cells that remained on coverslips after 9 or 10 days were grossly altered and there was total cell destruction after 11 days. At this stage of the infection there were plaques of fluorescence in both CSM-C and CSM-T infected cells stained with anti-CSM serum.

Fig. 34. Transmission electron micrograph of PK15 cells 8 days after infection with PIA filtrate containing CSM. Large numbers of bacterial forms (A) the majority of which are coccal in form and increased numbers of intracytoplasmic filaments (B) are evident.

(x 31250)

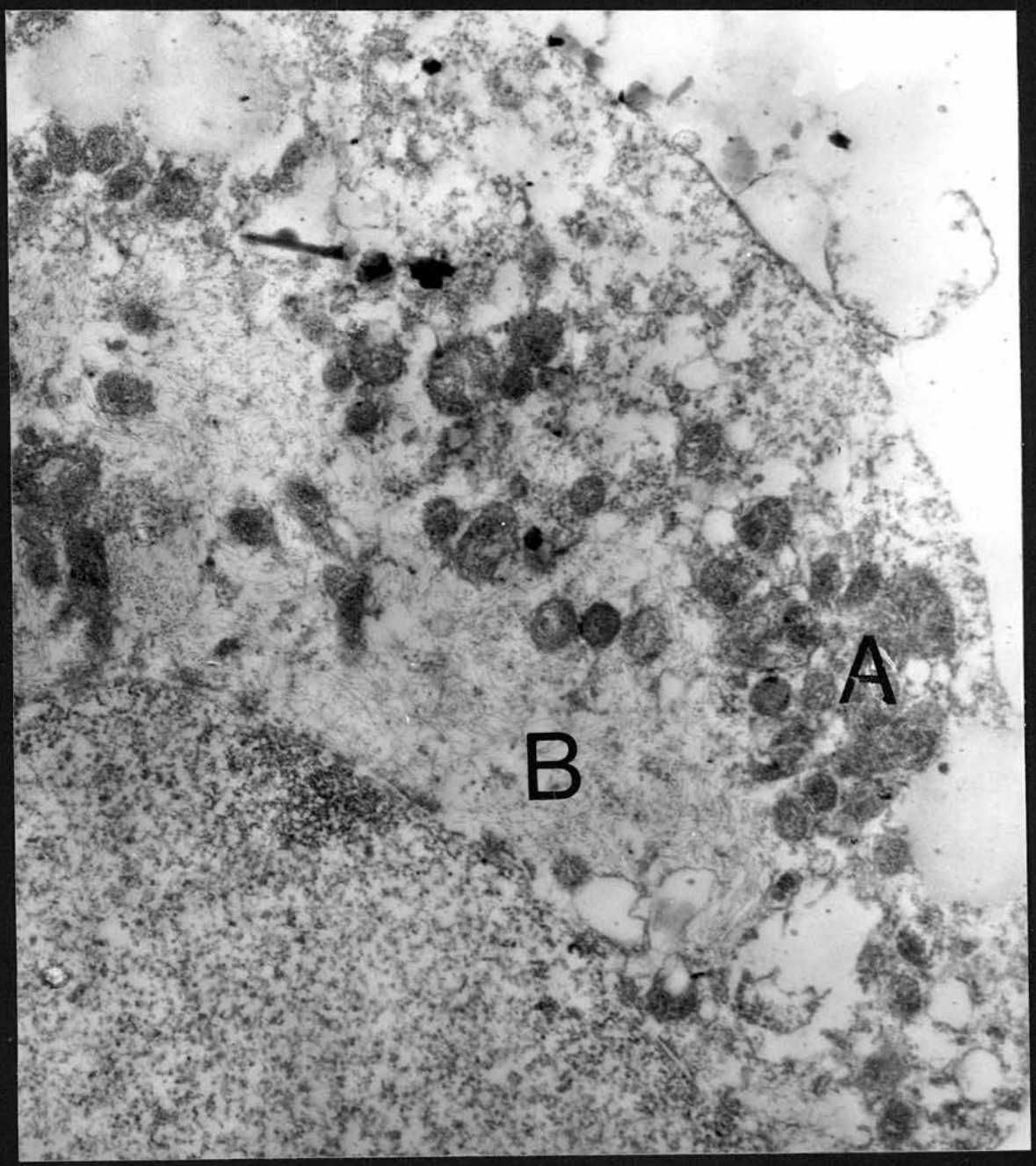

A
B

Table 29: Recovery of bacteria from supernatant fluids of PK15 cells infected with CSM-C or CSM-T and degree of bacterial parasitism

Time after infection in days	Bacterial counts as $\log_{10}$ organisms per ml from PK-15 cells infected with CSM-C		Filtrate from 363/78		Filtrate from 175/78	
0	10.30		4.70		3.48	
1	6.48	(94)	NR	(12)	NR	(9)
2	6.60	(95)	NR	(15)	NR	(12)
3	6.00	(95)	4.95	(21)	4.20	(18)
4	5.78	ND	5.30	(38)	4.48	(25)
5	5.40	(95)	6.48	(49)	5.30	(38)
6	4.70	ND	5.90	(58)	4.78	(48)
7	3.95	(97)	5.00	(79)	4.60	(61)
8	3.20	ND	4.30	(91)	4.00	(84)
9	2.40	(97)	3.70	(96)	3.70	(92)
10	1.70	ND	NR	ND	NR	ND
11	NR	ND	NR	ND	NR	ND

NR = No organism recovered

ND = Not examined

() = Numbers in brackets indicating percentage of cells showing intracellular fluorescence with anti-CSM serum.

7.5.4 Comments

The production of visible cytopathic changes by CSM-T inoculum that contained either 4.70 $\log_{10}$ or 3.48 $\log_{10}$ organisms per ml and the progressive intracellular parasitism shown in infected cells is important because these were not the results obtained with cells exposed to CSM-C inoculum of similar concentration. It will be recalled that in Chapter 3 an inoculum of cultured CSM that contained 5.26 $\log_{10}$ organisms per ml which gave a bacteria/cell ratio of 1:1, did not produce any visible cytopathic changes in PK15 cells after 13 days of exposure to infection and bacterial recovery from the supernatant fluids which decreased with time only could be detected over the first 3 days. Intracellular parasitism by bacteria was shown by only 11% of the cells and this decreased until fluorescence could not be detected after 11 days. The production of cytopathic changes by CSM-T inoculum as low as 3.48 $\log_{10}$ organisms per ml which gave a bacteria/cell ratio <1:1 suggests that such organisms are capable of growing rather more successfully in the intracellular location than culture derived bacteria. The high bacterial counts in supernatant fluids seen on day 5 - 9 in filtrate-infected cells may also indicate the presence of more viable bacteria in each infected cell as these raised counts were associated with lower levels of cell parasitism.

The cytopathic changes induced by either CSM-C or CSM-T in PK15 cells were similar in unstained and Giemsa-stained preparations. Ultrastructurally, some differences were shown by CSM-T infected cells namely: i) an increase in the number of intracytoplasmic filaments ii) a marked increase in the number of intracellular organisms reflected by the number of organisms recovered from the supernatant fluids after 3 to

6 days and iii) intracellular organisms did not show the characteristic outer, irregularly-formed, double, wavy layer of intracellular CSM-C. These increases in both intracellular and extracellular organisms did not speed up the destruction of the cell monolayers which took place after similar lengths of time in the three infections.

7.6 Immunofluorescence staining of CSM-C or CSM-T infected PK15 cells with antisera prepared against intracellular bacteria

7.6.1 Introduction

Previous immunofluorescence staining of cultured cells infected with CSM have employed hyperimmune rabbit "OH" antisera raised against CSM strain 253/72 and this serum has been used to demonstrate both intracellular and cell-surface associated bacterial antigen. Recently, Lawson *et al* (in press) immunised rabbits with bacteria extracted directly from the lesions of PHE and with it demonstrated an intracellular antigen (Ω) at the site of bacterial parasitism in the apical cytoplasm of cells of the lesions of PIA, PHE and in the lesions of hamster proliferative ileitis. These sera do not apparently react with any of the *Campylobacters* that have been isolated from the lesions of the porcine proliferative enteropathies in either agglutination or fluorescent antibody tests.

In this experiment PK15 cells infected with either cultured CSM or PIA derived CSM were stained with antisera from a rabbit immunised with the bacteria extracted from PHE tissues (RIΩS). This was an attempt to ascertain whether the infection of PK15 cells with CSM from either source will contain the intracellular antigen (Ω). Certain rabbit serum reacts also with the intracellular Ω antigen in immunofluorescent tests, the

particular anti-CSM (253/72) serum used in this test did not have this activity (Lawson, G.H.K., Rowland, A.C. and McIntyre, N (1985) Vet Microbiology 10, 303-313.)

7.6.2 Materials and Methods

Rabbit antiΩserum (1080/76) was supplied by Dr Lawson and prepared as described in Chapter 2.

Fluorescence staining with RIΩS was performed in parallel with anti-CSM serum on CSM-C and CSM-T infected PK15 cells (7.5.2). Briefly, day-old PK15 cells grown on coverslips were infected with either 24 hour-old CSM culture suspended in MEM, or 0.60 μm filtrate from PIA tissue. These infected cells were incubated at 37^{o}C, coverslips removed after 1 hour and thereafter daily for 11 days from each group. The coverslips were rinsed in warm PBS, fixed in acetone and stained by the immunofluorescence method as described in 2.9. Uninfected cells, and cultured CSM were stained separately as described and used as controls.

7.6.3 Results

Cells infected with either CSM-C or CSM-T demonstrated intracellular bright-stained fluorescing particles when stained with RIΩS after 1 hour of infection (Table 30). In both groups of infected cells there was an increase in the amount of particulate fluorescence observed with time throughout the 11 days of the experiment (Figs. 35-38). The antigen was present as coccoid bodies throughout the period of exposure of cells to CSM from either source. Infected cells stained with anti-CSM serum showed particulate antigens of both coccoid and vibrioid forms sometimes during infection (Fig. 39) but, to repeat, when stained with RIΩS demonstrated only coccoid

Fig. 35. PK15 cells 1 hour after infection with CSM-C. The intracytoplasmic brightly-stained particles are all coccoid in form.
Acetone fixation, immunofluorescence staining with RIΩS and sheep anti-rabbit (FITC) conjugate.
(x 180)

Fig. 36. PK15 cells 1 hour after infection with PIA filtrate containing CSM. Round brightly-stained intracytoplasmic particles can be seen.
Acetone fixation, immunofluorescence staining with RIΩS and sheep anti-rabbit (FITC) conjugate.
(x 180)

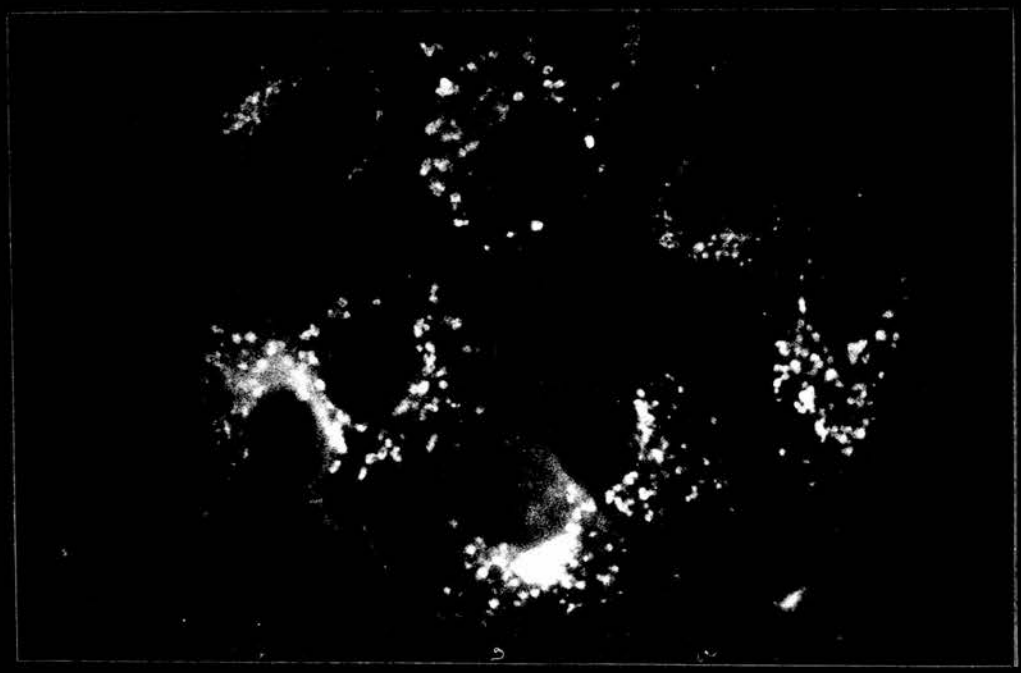
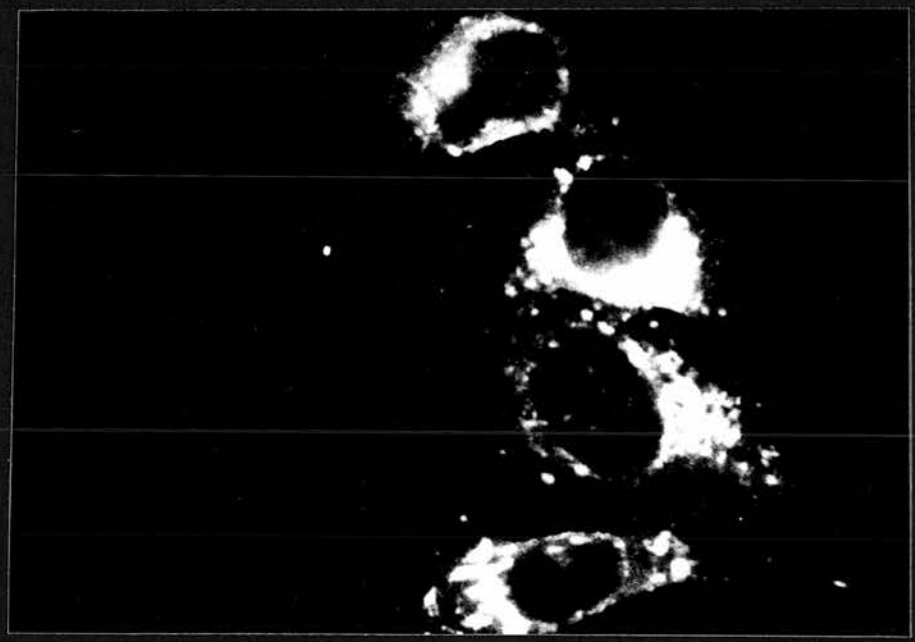
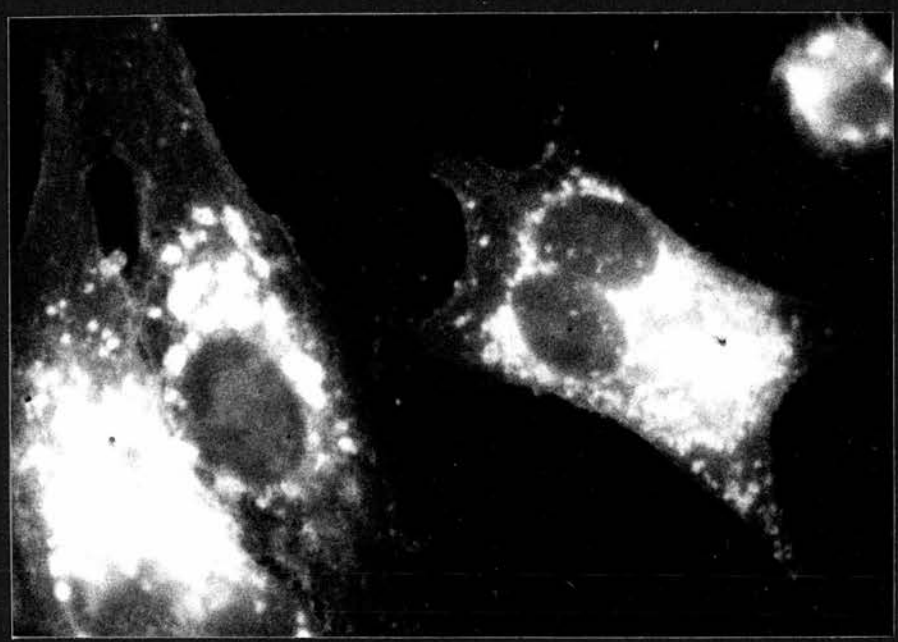


Fig. 37. PK15 cells 9 days after infection with CSM-C. Large numbers of intracytoplasmic coccoid particularly which appear as brightly stained areas are noticeable.
Acetone fixation, immunofluorescence staining with RIΩS and sheep anti-rabbit (FITC) conjugate.
(x 180)

Fig. 38. PK15 cells 9 days after infection with PIA filtrate containing CSM. Large numbers of intracytoplasmic fluorescing particles are seen.
Acetone fixation, immunofluorescence staining with RIΩS and sheep anti-rabbit (FITC) conjugate.
(x 284)

Fig. 39. PK15 cells 5 days after infection with PIA filtrate containing CSM. Intracytoplasmic antigen is present in both coccoid and vibrioid forms.
Acetone fixation, immunofluorescence staining with anti-CSM serum and sheep anti-rabbit (FITC) conjugate.
(x 284)





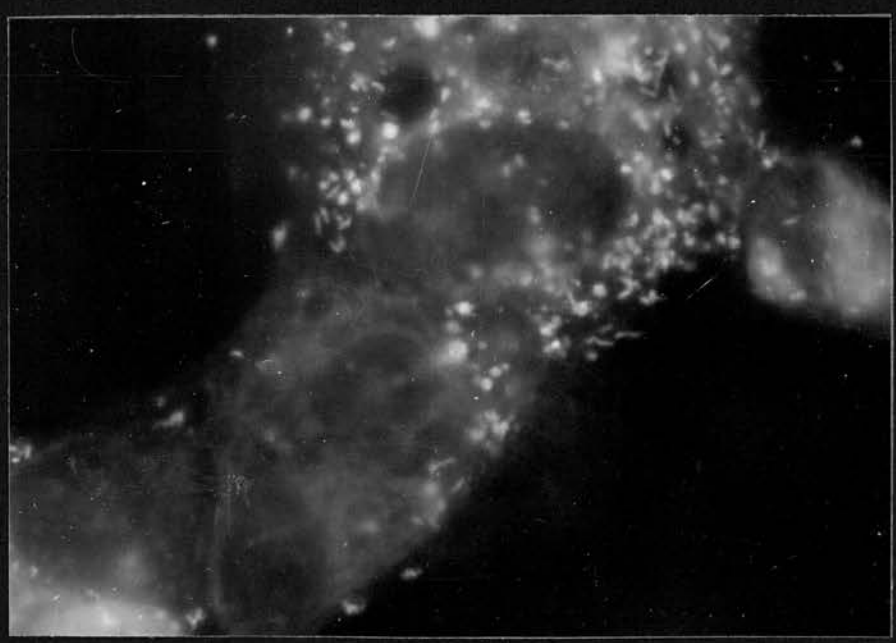


Table 30 Comparison of immunofluorescence staining of PK15 cells exposed to CSM-C or CSM-T for 1 hour using RIΩS or anti-CSM serum

Culture	Antiserum used	
	RIΩS	Anti-CSM serum
PK15 - not infected	-[a]	-
Cultured CSM	-	+
PK15 infected with CSM-C	+	+
PK15 infected with CSM-T	+	+

a Detection of antigen

- = antigen not detected

+ = antigen detected

forms throughout the experiment. Immunofluorescence stained preparations of uninfected PK15 cells, or cultured CSM did not show any fluorescence with RIΩS (Table 30).

7.6.4 Comments

There was no fluorescence in stained preparations using RIΩS of the uninfected PK15 cells, or cultured CSM, but cells infected with CSM from either source demonstrated intracellular particulate fluorescence. This result suggests that the antigen visualised in these cells might be the outcome of the cell infection with CSM from either source. The rapid appearance of the antigen in cells exposed to CSM infection may suggest that it is either an exposed or degraded bacterial antigen or possibly alternatively an altered host derived antigen on the bacterial surface. The appearance of Ω antigen in cells infected with CSM indicates that this bacteria may be a potential cause of the appearance of such antigen in the cells of PIA or PHE.

7.7 Examination of CSM-T inoculum for agents other than campylobacter and negative staining of CSM-C and CSM-T

7.7.1 Introduction

It has been demonstrated in experiment 7.5 that light microscopy showed similar cytopathic changes in unstained and Giemsa-stained preparations of CSM-C or CSM-T infected cells. There were differences in the pattern of bacterial recovery from the supernatant fluids of cells infected by bacteria from the two sources. The sudden increase in bacteria

in the supernatant fluids after 3 days in the cells infected with CSM-T either suggested that the two types of CSM (C or T) were behaving intrinsically in a different manner or that the culture contained another agent affecting the growth of CSM.

In this experiment the final filtrate was examined for the presence of other agents. Tissue-derived and cultured-CSM were also negatively stained in a search for any structural differences between bacteria derived from the two sources.

7.7.2 Materials and Methods

ii) Preparation of 0.45 μm filtrate from CSM-T inoculum

It will be recalled (7.3.5 (c)) that bacteria were not recovered from filtrates through 0.45 μm membranes. CSM-T suspension prepared as described in (7.3.5 (b)) was passed through a membrane filter of 0.45 μm APD by applying positive pressure. This bacteria-free filtrate was examined for the presence of virus particles.

ii) Negative staining of CSM-C, CSM-T or 0.45 μm filtrate

An overnight culture of CSM suspended in warm MEM and a filtrate of adenomatous homogenate passed through membrane filter of 0.60 or 0.45 μm APD (7.5.2) were stained as described below.

Some drops of each suspension were placed separately on a carbon-coated formvar film supported on a 400 mesh copper grid and after 30 seconds the excess fluid was removed by careful blotting. The grid was then rinsed in further drops of distilled water and the excess fluid removed by gentle blotting. The preparations were then stained for 10 - 20 seconds with one drop of one of the following: potassium phototungstate

(2% pH 6.3); potassium silicotungstate (2% pH 7.1); uranyl acetate (2% pH 4.5) or methylamine tungstate (Faberge and Oliver, 1974), (EM Scope Ltd., England). Excess stain was removed with good quality absorbent tissue and the preparation was held in an evacuated dessicator before being examined in a Philips 400 transmission electron microscope.

iii) Infection of PK15 cells with 0.45 μm filtrate

This cell infection served as a possible confirmation of the presence or absence of any viral agents in the 0.45 μm filtrate. If the number of viral particles present in the original filtrate were so few as not to be demonstrated by microscopy, their intracellular multiplication in cells might induce visible cytopathic changes.

Cells were grown on coverslips as monolayers for 24 hours and infected with 1 ml of the 0.45 μm bacteria-free filtrate prepared as in i) above. These were incubated at 37°C for 11 days. Every day a coverslip was removed and stained by Giemsa's method and viewed by light microscopy for any cytopathic changes. Uninfected coverslip monolayers were processed as above and used as controls.

7.7.3 Results

The only noticeable difference between bacteria derived from adenomatous tissue or culture was the presence of flagella on organisms derived from the latter but not from the former.

Negatively-stained preparations of 0.45 μm filtrate did not demonstrate any form of organisms and subsequent exposure of PK15 cells to the filtrate failed to induce any cytopathic changes in infected cells during the 11 days of the experiment.

7.7.4 Comments

Evidence suggests that motility of CSM plays a role in the attachment of bacteria to cells. Cells exposed to CSM derived from tissue did not show any bacterial attachment after 1 hour of infection (7.5.3). This may be as a result of CSM-T lacking flagellum immediately after separation from adenomatous tissue, which then regrow. High speed shearing is a well known method of removing flagella from bacteria (Montie et al, 1982; Holder et al, 1982) and homogenisation may act in this way. Alternatively, the relatively small numbers of bacteria in the filtrate may make it very difficult to observe bacterial attachment even if they attach to cells.

The CSM-T inoculum used in 7.5 did not demonstrate any viral agents since particles could not be demonstrated by negative staining of 0.45 μm filtrate and cells infected with this material remained as uninfected control cells throughout the experiment. These results are similar to the more intensive virological results reported in brief by Rowland and Rowntree (1972) which failed to detect virus from PHE cases. This suggests that the sudden increase in the number of bacteria in the supernatant fluids of CSM-T-infected cells after 3 to 5 days of infection (Table 29) was not as a result of any synergistic effect involving a viral or other agent in the filtrate but might be an innate characteristic of cell infection with bacteria derived directly from tissues.

7.8 Exposure of PK15 cells to bacteria extracted from PHE tissue

7.8.1 Introduction

A satisfactory method has been developed for the preparation of CSM derived from PIA tissue. Exposure of PK15 cells to this preparation resulted in cytopathic changes and bacterial multiplication (7.5).

Earlier workers (Love and Love, 1979; Lawson et al, 1979) have reported that although CSM may be isolated from PHE tissues, this is not always true and when it can be demonstrated to be present the viable organisms recovered are few in comparison with the numbers that may be isolated from PIA and that can be visualised intracellularly in PHE (Lawson, personal communication, 1983).

With the above in mind, this experiment involved the preparation of an inoculum from PHE tissue by a similar method to that for PIA tissue described previously (7.5.2). PK15 cells were then exposed to this filtrate to find out whether it induced cytopathic changes in infected cells in the same way as PIA filtrates.

7.8.2 Materials and Methods

Aliquots of PHE tissues, 1080/76 and 1269/76 (Table 25) were used for this experiment.

i) Preparation of bacterial inoculum from PHE tissue

All processes from the weighing of PHE material to the final filtration through 0.6 μm membrane were performed as for PIA material previously described in 7.5.2.

ii) Growth of cells used for infection

PK15 cells were grown on coverslips in 2 ml screw-capped vials for 24 hours as described in 7.5.2.

iii) Infection of cells with 0.60 or 0.65 µm filtrate

Coverslip monolayers of PK15 cells grown for 24 hours (ii) were exposed to 1 ml of 0.60 or 0.65 µm filtrate at 37°C and then incubated for 11 days. Infected coverslips were removed after 1 hour and thereafter daily, processed for Giemsa and immunofluorescence staining.

Culture of bacteria in the supernatant fluids of infected cells was also attempted daily by spreading 0.1 ml of the fluid on dried duplicate CBA plates and incubating at 37°C in a microaerophilic or aerobic atmosphere for 48 hours. Uninfected coverslips were removed, processed as above and used as controls.

7.8.3 Results

The 0.6 µm filtrate used as inoculum did not yield bacteria by culture on CBA plates and cytopathic changes in PK15 cells did not take place. A second experiment using a 0.65 µm filtrate as inoculum showed that it contained one bacterial type. In these two exposure experiments Brucella differential stained preparations of the trypsinate, homogenate or filtrates of the PHE tissue showed bright red stained campylobacters. The bacteria contained in the 0.65µmfiltrate were delicate Gram-ve curved rods catalase positive which grew as greyish, round, convex, smooth colonies on CBA under microaerophilic conditions, did not grow aerobically and were not agglutinated by anti-CSM (253/72) serum. These campylobacters had the characteristics of C. coli or C. jejuni (Lawson et al, 1979). This organism

multiplied in the extracellular fluid of cells infected with 0.65 μm filtrates and after 24 hours of infection the supernatant fluid was yellow in contrast to uninfected controls whose fluids were reddish-purple. After 2 days the monolayers of the infected cells were totally destroyed and detached from the coverslips. Giemsa and immunofluorescence stained preparations of cells 1 hour after exposure to infection did not show any bacterial attachment or fluorescence with anti-CSM serum respectively.

7.8.4 Comments

Despite the visible presence of large numbers of bright red campylobacters in Brucella differential stained preparations of the tissue, organisms similar to CSM could not be recovered from the tissue at any stage of the preparation of the inoculum. If the bright red stained organisms in PHE tissue are CSM then these results suggest that they are either CSM which cannot grow in culture or that they are a different and as yet unisolated campylobacter. Since both 0.65 and 0.60 μm filtrate prepared from PHE tissue demonstrated red stained organisms and *C. coli/jejuni* could only be recovered from 0.65 μm filtrates this indicates that either these isolates are not the red stained organisms or that the bright red organisms in 0.60 μm filtrate are possibly similar or different damaged bacteria (as suggested by Roberts, 1978) which are unable to grow in culture. Alternatively, the red-stained organisms may be distinct from any of the campylobacters isolated to date and are unable to grow in the media and atmosphere used. It is most likely therefore that these bright red-stained bacteria cannot grow in culture. Previous attempts by Lawson *et al* (1979) to grow these organisms in eggs as well as synthetic media only resulted in irregular recovery of small numbers of bacteria. This present

work now indicates that these campylobacter-like forms seen in PHE cases will not grow in pig kidney cell lines and further strengthens the case that these are damaged or non-viable campylobacters.

7.9 Attempts to passage CSM cell infection derived from adenomatous tissues

7.9.1 Introduction

It has been demonstrated that cultured CSM could not be passaged more than twice in PK15 cells irrespective of whether the source of inoculum was lysed infected cells or the supernatant fluid (Chapter 6). Experiment 7.5 showed some differences between the behaviour in PK15 cells of CSM derived from adenomatous tissue and cell free cultures.

This experiment was designed to examine whether bacteria derived directly from adenomatous tissue could be passaged in PK15 cells in a further search for differences between CSM-C and T and to examine whether another campylobacter-like bacteria could be growing in the PK15 cells along with CSM following infection with PIA suspensions.

7.9.2 Materials and Methods

This experiment was a continuation of the primary infection of PK15 cells with CSM derived from PIA tissue in 7.5.2.

i) Passage in PK15 cells of CSM-T using lysed infected cells

Only cells infected with filtrate from PIA tissue (363/78) were used in this propagation experiment.

After 5 days of the primary infection of cells grown in medical flats the monolayer was washed vigorously to eliminate extracellular bacteria

as previously described in 5.2.1. The washed cells were lysed with 1% Nonidet P40 as described in 5.2.3 and used to infect 24-hour-old PK15 cells grown on coverslips or in medical flats as appropriate as previously described (6.2.1). Cells were incubated at 37°C for 11 days. Every day infected coverslips were removed, rinsed and processed for Giemsa and immunofluorescence staining as previously described (Chapter 2). Uninfected coverslips were removed, processed as above and used as controls. The whole process was repeated after 2 days of the first passage.

ii) Passage of CSM-T from supernatant fluids of infected PK15 cells

This was a parallel experiment to infection with CSM from lysed infected cells. Before washing and lysing infected cells as in i) the supernatant fluids were collected and used to infect 24-hour-old cells grown on coverslips or in medical flats by a similar method as described in 6.3.2. These were incubated at 37°C for 11 days. Coverslips were removed daily and processed for Giemsa and immunofluorescence staining.

7.9.3 Results

i) Passage of CSM from lysed infected cells

PK15 cells exposed to lysed infected cells yielded decreasing numbers of CSM from the supernatant fluids during the initial 3 days of the first passage, and also the amount of intracellular antigen demonstrated by immunofluorescence staining fell from the first to the fifth day of infection. In the second passage bacteria were neither recovered from the supernatant fluids nor seen within the cells (Table 31a). Cells exposed to lysed infected cells did not show any cytopathic changes throughout the serial passages.

Table 31a Passage of CSM from lysed CSM-T-infected PK15 cells: recovery of bacteria from supernatant fluids and the number of cells showing intracellular fluorescence.

Time after infection in days	1st Passage		2nd Passage	
	Bacterial count in supernatant fluids as $\log_{10}$ organisms per ml	Percentage of cells showing intracellular fluorescence	Bacterial count in supernatant fluids as $\log_{10}$ organisms per ml	Percentage of cells showing intracellular fluorescence
0	3.26		2.23	
1	2.08	8	NR	0
2	2.23	ND	NR	0
3	1.30	3	ND	ND
4	NR	ND	ND	ND
5	NR	1	ND	ND
6	NR	ND	ND	ND
7	NR	0	ND	ND
8	NR	0	ND	ND

NR = No organisms recovered

ND = Not examined

Table 31b Passage of CSM from supernatant fluids of CSM-T-infected PK15 cells: recovery of bacteria from supernatant fluids and number of cells showing intracellular fluorescence

Time after infection in days	1st Passage		2nd Passage		3rd Passage	
	Bacterial counts in supernatant fluids as $\log_{10}$ organisms per ml.	Percentage of cells showing intracellular fluorescence	Bacterial counts in supernatant fluids as $\log_{10}$ organisms per ml.	Percentage of cells showing intracellular fluorescence	Bacterial counts in supernatant fluids as $\log_{10}$ organisms per ml.	Percentage of cells showing intracellular fluorescence
0	6.30		4.95		2.98	
1	4.88	15	2.90	6	NR	0
2	4.95	ND	2.98	ND	NR	0
3	4.23	10	1.85	3	NR	0
4	3.67	ND	NR	ND	ND	ND
5	2.86	7	NR	1	ND	ND
6	1.98	ND	NR	ND	ND	ND
7	NR	4	NR	0	ND	ND
8	NR	ND	NR	0	ND	ND
9	NR	1	NR	ND	ND	ND
10	NR	ND	ND	ND	ND	ND
11	NR	0	ND	ND	ND	ND

NR = No organisms recovered

ND = Not examined

ii) Passages of CSM from supernatant fluids of infected cells

PK15 cells exposed to the supernatant fluids of infected cells in the 1st and 2nd passages showed a similar decrease in the number of organisms recovered from the fluids and the number of intracellular bacteria demonstrated as the days of infection increased. In the 1st passage bacteria were demonstrated in infected cells 9 days after infection and organisms were recovered from the supernatant fluids up till 6 days after infection. In the 2nd passage bacteria were recovered from the supernatant fluids for 3 days after infection and intracellular bacteria was demonstrated for 5 days. In the 3rd passage organisms were neither recovered from the fluids nor demonstrated within cells (Table 31b). There were no visible cytopathic changes in the cells during the serial passages.

7.9.4 Comments

In both experiments where cells had been infected with the lysate or supernatant fluids of infected cells there was a similar decrease in the number of CSM recovered from the fluids and demonstrable intracellular bacteria. Although the supernatant fluid from the primary infection of PK15 cells with CSM-T used as inoculum contained 6.30 $\log_{10}$ organisms per ml, it has behaved similarly to CSM-C or CSM-TC in these propagation experiments. Until a much higher infecting dose is obtained from infected cells continuous culture will probably not exceed 3 passages in PK15 cells. The low infecting doses of bacteria from these three sources have contributed to the failure of organisms to persist and multiply inside the cell and this affects the number of passages of CSM possible. However, the elimination of CSM and lack of cytopathic changes on subculture in PK15 infected with PIA filtrates is further evidence that i) there is an absence of CPE

producing virus capable of infecting PK15 cells and ii) there is an absence of either campylobacter-like organism that might have multiplied in the cells along with CSM. This experiment once again emphasises the essential difference in the behaviour of tissue derived CSM to that obtained from other sources, tissue culture or cell free media.

7.10 Discussion

The use of antibiotic supplement (x 2) in the culture medium, the passage through membrane filters of different APD and the dilution of filtrate were successfully used to obtain a final inoculum that contained apparently one bacterial type that showed all the characteristics of CSM and the intracellular campylobacter-like organism in PIA. The problem of multiplication of bacteria in the extracellular fluid in the study of interactions between bacteria and cells in culture was highlighted by the results obtained after exposing PK15 cells to either of two isolates (type 1 or 2) contained in filtrates from PIA or that from PHE tissue. Even the presence of antibiotics in the growth medium did not prevent the multiplication of these organisms in the extracellular fluid.

Light microscopy of Giemsa-stained and unstained preparations of cells infected with either CSM-C or CSM-T showed that bacteria from both sources produced similar cytopathic changes in cells. However, transmission electron micrographs of CSM-T-infected cells showed an increase of intracytoplasmic filaments during the later stages of the infection. This increase has been noted in: chondrocytes of rabbit articular cartilage after experimentally produced chronic haemarthrosis (Roy, 1968), synovial intimal cells in rheumatoid arthritis (Ghadially and Roy, 1967), chondrocytes of rabbit articular cartilage associated with ageing (Barnett et al, 1963). The general

opinion of these workers is that an increase in intracytoplasmic filaments in these cells is a sign of regressive or degenerative changes engendered by age or noxious influences. The idea that an increase in filaments is a degenerative change is also supported by the observation that there is an increase in filaments in human lens epithelial cells related to ageing and pathological conditions (cataract) (Perry et al, 1979). Thus, reports by these workers show the evidence which indicates that a gross increase in intracellular filaments is a sign of regressive and degenerative changes in a variety of cell types which has also been demonstrated in PK15 cells exposed to CSM-T but not to CSM-C.

It has been demonstrated that the inoculum prepared from PIA tissue contained apparently only one bacterial type which had the characteristics of CSM. Other bacterial types were recovered from homogenised PIA tissue in which type 2 was the only catalase positive organism. Catalase-positive campylobacters C. coli, C. jejuni, C. fetus ss fetus or C. hyointestinalis have been isolated from the intestines of pigs with proliferative enteropathy (Lawson and Rowland, 1974; Rowland and Lawson, 1975; Gebhart et al, 1983). Rajasekhar (1981) reported that primary and established pig kidney cells infected with C. hyointestinalis (124/73 A4) showed degenerative cytopathic changes after 24 hours of infection and by the second day there was total destruction of the cells. Newell and Pearson (1981) working with HeLa 229 and Int 407 cells showed that exposure of these cells to C. jejuni for 18 hours produced significant numbers of dead and dying cells evidenced by the presence of cell-surface blebs, pits and crevices on scanning electron micrographs of the infected cells. These results show how rapidly these catalase-positive campylobacters can induce cytopathic changes in cultured cells. Organisms recovered from PIA except type 3 produced similar effects in PK15 cells type 1 isolates which was not

a campylobacter and Campylobacter sp. (type 2) both multiplied in the extracellular fluid and rapidly destroyed the cells within 2 days.

The 0.60μm filtrate from PIA showed bright red stained organisms and only one bacterial colony type (3) similar to cultured CSM was recovered from it and this suggested that the bright red organisms were CSM or that the filtrate contained an additional organism which could not be cultured. Evidence has shown that there were neither virus particles nor campylobacter-like organisms present in the 0.45 μm filtrates, so some of the bright red organisms were CSM. The inability to reproduce the disease in gnotobiotic animals using cultured CSM might therefore be due to alteration in the pathogenicity of CSM-C through repeated subculturing. Bacteria used for infection of gnotobiotic piglets had however only been subcultured the minimum number of times (McCartney et al, 1984). An inoculum of 3.48 $\log_{10}$ organisms per ml of tissue-derived CSM initiated infection in PK15 cells which was not possible with an infecting dose of 5.26 $\log_{10}$ organisms per ml of cultured CSM; this observation appears to justify the proposition that tissue derived CSM might have more virulence for experimental animals than culture derived organisms.

The sudden increase after 3 days of infection in the number of organisms in the supernatant fluids of cells infected with tissue-derived CSM which did not spread up cell destruction, appears to be a characteristic of CSM-T and related to its infectivity, or pathogenicity. There was no evidence that this increase was the result of the presence of another organism in the filtrate that could affect CSM growth. Tissue-derived CSM infection could not be serially propagated in PK15 cells for after the primary infection with CSM-T both the recovery of organisms from the supernatant fluids and production of CPE in subsequent passages were similar to those of PK15 cells infected with low inoculum of CSM-C. It

appears therefore that once CSM-T has been used to infect cells it loses its "virulence" and this together with the low (<7.0) number of organisms in the inoculum affects attempts to serially passage CSM-T infection in PK15 cells. Similar results have been reported by Jacoby and Johnson (1981) in cell cultures of CER which were infected with mucosal suspensions of affected hamster ilea. Infected cells remained viable for up to 3 weeks and contained intracellular campylobacter-like forms yet conventional bacteria were not demonstrable. These cells which were passaged at weekly intervals ceased to be infectious for new CER cultures after 2 passages.

Experiment 7.8 failed to demonstrate that the intracellular organisms present in PHE tissues were other than CSM and the most convincing explanation is that the bacteria have been possibly altered and cannot be recovered on conventional media as postulated by Lawson et al (1979). CSM can be recovered on occasion from the lesions of PHE in numbers similar to those observed (Lawson et al, 1979) in tissues, and in small numbers from some cases (Lawson et al, 1979; Love and Love, 1979). The presence of immunoglobulin A (IgA) or Ω antigen present in cells may interfere with the recognition of specific bacterial antigens.

The inoculum from PHE tissues contained catalase-positive organisms with the characteristics of Campylobacter coli/jejuni group which multiplied in the extracellular fluids of infected tissue culture cells. Small numbers of Campylobacter sp. were also isolated from PIA tissue, these two isolates from PHE and PIA multiplied in the extracellular fluids of infected cells and caused the destruction of PK15 cells within 2 days of infection. It is not possible to exclude such organisms as the cause of PIA or PHE but the evidence is not convincing and infection of pigs with C. coli (Taylor and Olubunmi, 1981) does not substantiate such a claim.

It has been shown by Lawson et al (in press) that serum from rabbits immunised with bacteria extracted from the lesions of PHE reacts with intracellular Ω campylobacter-like antigen present in the cells of the proliferative enteropathies of the pig and the hamster. Jacoby and Johnson (1981) have reported that 1 week post-inoculation of primary hamster embryo cells with suspensions of affected hamster ilea, the cells contained intracytoplasmic rod-shaped bacteria that reacted with anti-TIH serum but not with normal serum or hyperimmune serum to conventional intestinal organisms. It appears that a similar Ω antigen is present in PK15 cells infected with CSM-C or CSM-T even after only 1 hour of infection. At this stage the majority of the bacteria are vibrioid and still attached to the cell surface whilst the fluorescing particles are coccoid and are not attached to the periphery of the cells.

CHAPTER 8.

Chapter 8

Long term studies of pig kidney (PK15) cells infected with cultured CSM

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8.1 General Introduction

Experimental evidence has demonstrated that about 5% of the cells of monolayers of PK15 cells infected by CSM did not show any bacterial attachment after 1 hour nor appeared to contain demonstrable intracellular bacteria after 24 hours of infection. In theory, these few refractory cells should remain apparently normal whereas, in practice, there was total destruction of all cells of the monolayer after 10 days of infection due, possibly to the prolonged exposure of the cells to the metabolic products of infected cells and intracellular bacteria. This short period of time that infected PK15 cells survive make them unsuitable for long term studies of infection.

Rajasekhar (1981) showed that there is a marked variation in the response of different types of cell cultures to CSM infection. This includes attachment, the ability to support parasitic growth, susceptibility to lethal effects of parasitism and the type of cytopathic changes. Cell lines that persist despite intracellular parasitism could be of value since they may bear some resemblance to the tissue parasitism observed in field cases of proliferative enteropathies. Rajasekhar (1981) tried to establish a continuously infected line of abnormal pig kidney cells, but found that even the addition of 20-30% of foetal calf serum whilst prolonging cell attachment did not enable the cells to multiply.

PK15 cells were the least refractory to CSM infection among the cultured cells tested by Rajasekhar (1981) and being of pig origin are the closest readily available eell line to the cells involved in the natural

disease; attempts were therefore made to establish a line of continuously infected PK15 cells with the aim of examining long term infection of such cells by CSM.

8.2 Attempts to establish a cell line of CSM-infected pig kidney cells (PKmi)

It will be recalled that the experiments described in Chapter 5 showed that vigorous washing of CSM-infected PK15 cell monolayer eliminated extracellular bacteria. In this experiment the same technique was used in the early stages of the infection to eliminate extracellular organisms, since prolonged exposure of the cell monolayer to supernatant fluid containing bacteria possibly contributes to the total destruction of the monolayer.

8.2.1 Materials and Methods

Cell washing of infected PK15 cells was started early after infection and before cytopathic changes were evident, in an attempt to allow non-infected or lightly infected cells to survive.

i) Growth and infection of PK15 cells with CSM

The infection of 24-hour-old monolayers of PK15 cells grown on coverslips and in medical flats was performed as previously described in 7.5.2.

ii) Serial passage of infected cells

After 3 days of infection of cells grown in medical flat the supernatant fluid was discarded and the monolayer vigorously washed with warm MEM as previously described (5.2.1). The cells were then detached from glass with 10 ml STV centrifuged at 200 x g for 1 minute and the pellet resuspended in growth MEM. The cells were then dispensed into test tubes with coverslips and medical flats in either 1 ml or 10 ml amounts as appropriate at concentration of 1 x 10^5 cells per ml and incubated at 37°C. The monolayers were subcultured after trypsinisation at weekly intervals, refed with maintenance medium every 3 days, and examined over a period of 14 weeks. Uninfected cells were subcultured as above and used as controls. Each week, prior to splitting of cultures, 0.1 ml of the supernatant fluids was inoculated on to duplicate CBA plates which were incubated microaerophilically for 48 hours at 37°C and examined for the presence of bacteria. Coverslips of infected and uninfected monolayers were removed at weekly intervals rinsed in warm PBS and stained by immunofluorescence or Giemsa's method. Some coverslips were processed for transmission electron microscopy by methods previously described in Chapter 2.

8.2.2 Results

i) Primary infection, 1st and 2nd passages (0 - 17 days)

In the primary infection during the first 3 days cells showed cytopathic changes similar to those described in Chapter 3 for PK15 cells exposed to CSM. Bacteria were not recovered from the supernatant fluids of subcultured infected cells in the 1st or 2nd passages. About 95% of cells demonstrated intracellular fluorescence when stained with anti-CSM serum during the first 3 days of the primary infection and a similar number of cells showed

brightly stained bacterial antigens in the 1st and 2nd passages (Fig.40). The percentage of cells showing cytopathic changes decreased from about 30% in the primary infection after 3 days to about 20% of the cells in the 1st or 2nd passages. During this period transmission electron microscopy demonstrated intracellular organisms but no noticeable effects on cytoplasmic organelles or the nucleus.

ii) Third to 7th passage (18 - 52 days)

Bacteria were not recovered at any time from the supernatant fluids of subcultured infected cells at the 3rd to 7th passage levels. The number of cells showing brightly stained bacterial antigens decreased to about 25% by the 7th passage . There was a marked increase in the number of apparently normal cells. Transmission electron micrographs showed few intracellular bacteria in each of the passages (Fig.41).

iii) Eight to 14th passage (53 - 101 days)

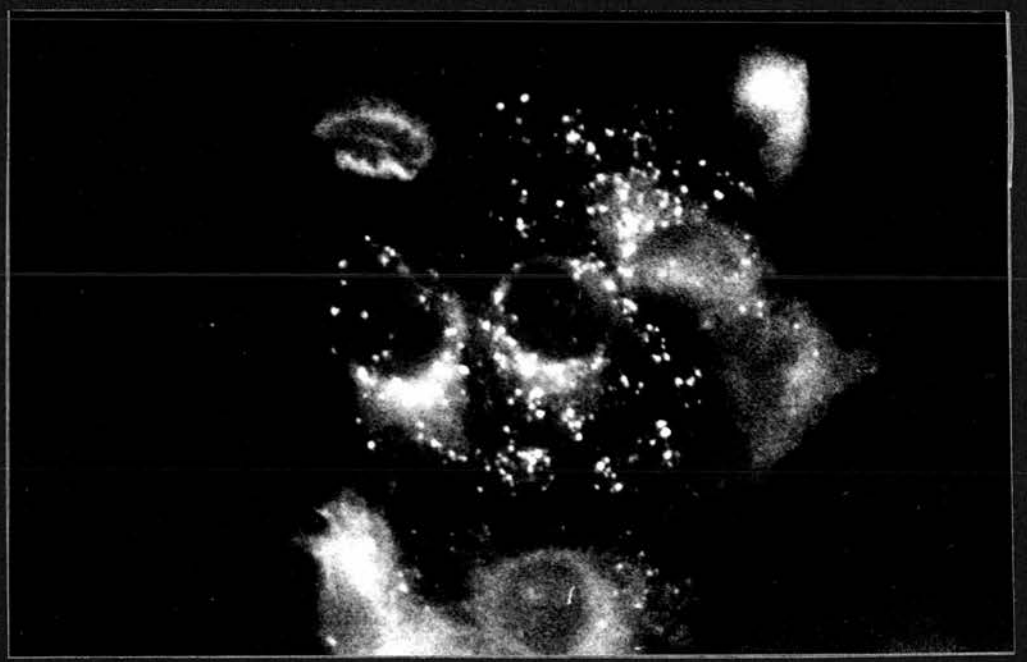
Examinations of Giemsa-stained cell cultures showed almost complete absence of abnormal cells although occasional cells with enlarged nuclei were present (Fig.42). The monolayers became more or less confluent and cells grew rapidly without showing cytopathic changes. Bacteria were not recovered from the supernatant fluids of these cell cultures. Immunofluorescence staining showed a progressive decrease in the number of cells with intracellular bacterial antigens up to the 10th and subsequent passages from which time no fluorescence could be demonstrated (Fig.43). Transmission electron micrographs also failed to show any intracellular bacteria.

Fig. 40. Passage of washed PK15 cells, 10 days post infection with CSM-C, first passage. Brightly stained particulate coccoid bacterial antigens are present in the cytoplasm.
Acetone fixation, immunofluorescence staining with anti-CSM serum and sheep anti-rabbit (FITC) conjugate.

(x 180)

Fig. 41. Passage of washed PK15 cells 38 days post infection with CSM-C, fifth passage. Transmission electron micrograph showing intracellular bacteria-like forms (arrowed).

(x 19250)



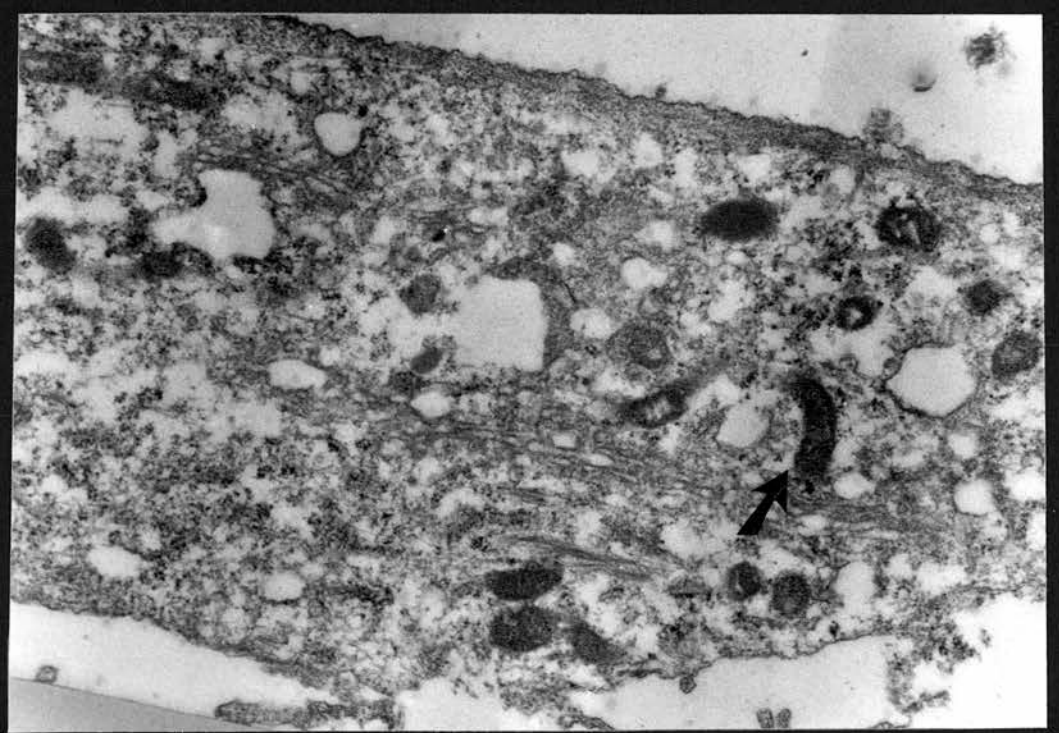
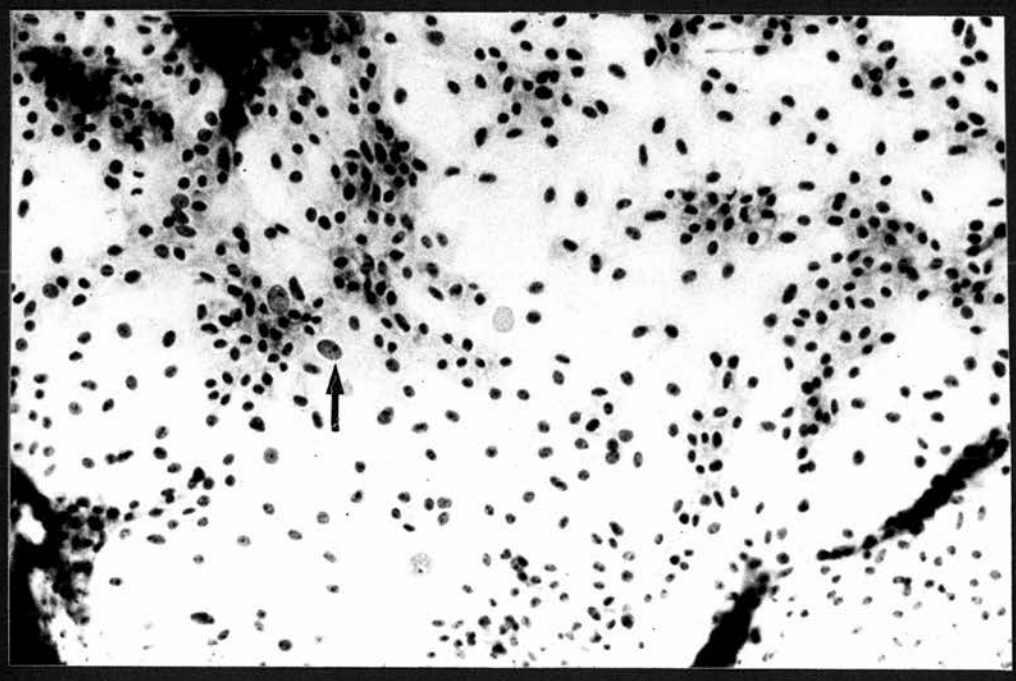


Fig. 42. Passage of washed PK15 cells, 93 days post-infection with CSM-C, 13th passage. Occasional cells with enlarged nuclei (arrowed) can be seen.

Giemsa stain (x 45)

Fig. 43. Passage of washed PK15 cells, 93 days post-infection with CSM-C, 13th passage. Bacterial antigen are absent from cell monolayer.
Acetone fixation, immunofluorescence staining with anti-CSM serum and sheep anti-rabbit (FITC) conjugate.

(x 284)



8.2.3 Comments

This experiment has confirmed the inability of CSM to infect all the cells in a population or to persist in infected cells over several serial passages. The inability to demonstrate organisms in both immunofluorescence-stained preparations and transmission electron micrographs of CSM infected PK15 cells after 93 days is interesting in that the time scale appears approximately to be similar to the events in infection of experimental animals with homogenised affected mucosa (Roberts et al, 1977) and in the natural disease.

The cell line that had survived this infection (PKmi) was used for subsequent experiments; these were once-infected PK15 cells, no longer yielding CSM and not showing any residual bacterial antigens.

8.3 Comparison of the attachment of CSM to PK15 and to PK15 cells previously exposed to infection (PKmi)

8.3.1 Introduction

A cell line (PKmi) was established as described in the last experiment (8.2). It is not known whether the total absence of bacterial antigen in these cells relates to death of all the infected cells in the population with subsequent replacement by non-permissive cells or whether the washing procedure allows infected permissive cells to survive the infection. If the former is the case then infection of PKmi cells will not show any bacterial attachment but if the latter is true then there will be some bacterial adherence to cells.

In this experiment PKmi and PK15 cells were each infected with CSM

to compare bacterial attachment.

8.3.2 Materials and Methods

i) Preparation of bacteria inoculum

Bacterial suspension of CSM strain 253/72 was prepared from 24 hour-old CBA slope cultures as described previously in Chapter 2.

ii) Inoculation of cell cultures

Day-old preformed coverslip cultures of PK15 or PKmi cells obtained by routine cell culture procedures were overlaid with 1 ml of the suspension of CSM and incubated at 37°C. Coverslips were removed at hourly intervals during the first 4 hours, and thereafter at 24 and 48 hours post-infection. After thorough rinsing in several changes of warm PBS to remove unattached bacteria, coverslip monolayers were fixed in methanol and stained by Giemsa's method.

8.3.3 Results

Giemsa-stained preparations of infected cells showed that the degree of attachment of CSM to PK15 and PKmi cells were similar (Fig.44) but the percentage of cells showing bacterial attachment differed during the first 4 hours of infection (Table 31). There appeared to be a greater proportion of non-permissive cells in PKmi than in PK15 cell population. These differences were significant as shown by t-test, $t = 17.16$ $p < .001$ for the first 4 hours of the experiment. Organisms were not demonstrated on the cell surfaces of either cell type after 24 or 48 hours of infection.

Table 31 Attachment of CSM to PK15 and PKmi cells

Time after infection in hours	Degree of attachment to cell cultures			
	PK15		PKmi	
1	+++	(95)	+++	(88)
2	+++	(95)	+++	(87)
3	+++	(94)	+++	(87)
4	+++	(90)	+++	(84)
24	-	(0)	-	(0)
48	-	(0)	-	(0)

\- = No bacteria attached to cell surface

+++ = Most of the cells showing 20 or more attached bacteria per cell

( ) = Numbers in brackets indicating the percentage of cells showing bacteria attached to the cell surface

8.3.4 Comments

The results indicate that the PKmi cell population did not arise from only non-permissive cells but is made up of both permissive and non-permissive cells. Rajasekhar (1981) has shown that CSM does not attach to the surface of pig kidney cells recently infected by CSM. CSM attached to PKmi cells which suggests that the receptor sites for bacterial adhesins are replaced sometime after attachment and the elimination of infection. The receptor sites appeared to be similar in both cell types, hence the similarity of bacterial adherence. The apparent increase in non-permissive cells may indicate that the infection with CSM has resulted in an altered cell population that differs metabolically from the original PK15 line.

8.4 Cytopathic changes induced following exposure of PK15 and PKmi cells to CSM

8.4.1 Introduction

It has been shown in Chapter 3 that a standard inoculum of CSM produced cytopathic changes in PK15 cells. In the last experiment (8.3) it was demonstrated that the PKmi cell population contained more non-permissive cells than the parent PK15 population. For this reason, the following experiment was undertaken to examine the effect of a standard inoculum of CSM on the cytopathic changes in PKmi and in PK15 cells.

8.4.2 Materials and Methods

PK15 and PKmi cells, grown separately as monolayers on coverslips for 24 hours, were infected with 1 ml of a 24 hour-old culture of CSM suspended in warm MEM and then incubated at 37°C for 14 days. Every day coverslips were removed from the infected cell cultures rinsed in warm PBS fixed in methanol or acetone and stained by either Giemsa's or immunofluorescence methods as appropriate. The number of organisms in the supernatant fluids of infected cells were also determined daily by the surface viable count method as previously described. Uninfected PK15 and PKmi coverslip monolayers were also processed as described and used as controls.

8.4.3 Results

The cytopathic changes produced by CSM in PKmi cells throughout the experiment were similar to those described for infected cells in Chapter 3. After 24 hours of infection there was a decrease in the number of organisms in the supernatant fluids of both infected cells which was more marked in PKmi. There were no visible cytopathic changes in either infected PK15 or PKmi cells. In both systems organisms were not demonstrated on the cell surface after 24 hours of infection and thereafter. Only 88% of the cell population of infected PKmi showed intracellular fluorescence compared with 95% in infected PK15 cells (Table 32).

There were no visible cytopathic changes in Giemsa-stained monolayers of infected PKmi cells after 2 days of infection while infected PK15 cells showed perforations in the cell sheet with about 10% of the cells rounding up and these abnormal cells showed granularity of the cytoplasm. A few cells had apparently started to fuse at this time.

Table 32 Exposure of PK15 and PKmi to CSM: recovery of bacteria from supernatant fluids, intracellular parasitism and severity of cytopathic changes produced in cells.

Time after infection in days	PK15			PKmi		
	Bacterial counts of supernatant fluids as $\log_{10}$ organisms per ml.		Severity of CPE	Bacterial counts of supernatant fluids as $\log_{10}$ organisms per ml.		Severity of CPE
0	10.74			10.74		
1	6.24	(95)	0[a]	5.60	(88)	0
2	6.35	ND	1	5.70	ND	0
3	6.00	(95)	2	5.18	(88)	1
4	5.78	ND	2	4.78	ND	1
5	5.30	(95)	3	4.38	(89)	2
6	4.60	ND	3	3.70	ND	2
7	4.00	(96)	4	2.88	(89)	3
8	3.48	ND	4	1.88	ND	3
9	2.88	(97)	5	NR	(89)	4
10	1.70	ND	5	NR	ND	4
11	NR	ND	6	NR	ND	5
12	ND		ND	ND		5
13	ND		ND	ND		6
14	ND		ND	ND		

ND = Not examined

NR = No bacteria recovered

0 = No CPE

a = Increasing severity of CPE: 1 = detectable CPE, 6 = complete cell detachment from coverslips

( ) = Numbers in brackets indicating the percentage of cells showing intracellular fluorescence.

Visible cytopathic changes started to show after 3 to 4 days of infection in PKmi cells when gaps appeared in the monolayer. There was rounding up of some cells and Giemsa-stained preparations showed a few enlarged nuclei and a few cells contained 2-3 nuclei. Some cells had small vacuoles towards the periphery of the cells. Cytopathic changes in infected PK15 cells progressed with focal destruction of the monolayer, increased cellular granularity, vacuolation of the cytoplasm and rounding up of cells. About 30% of the cells had enlarged nuclei and there was cell fusion resulting in polykaryons containing 5-6 nuclei. There was a progressive decrease with time in the numbers of organisms in the supernatant fluids of both infected cell types.

After 5-9 days of infection the monolayers of infected PK15 cells contained cells with immensely enlarged cytoplasm and nuclei. There was progressive destruction of the monolayer with polykaryons containing 15-20 nuclei of varying sizes and extensively vacuolated cells all remaining in patches on the coverslips. Cellular destruction progressed in infected PKmi cells with the supernatant fluids turning acidic and cloudy. More cells remained attached and of normal morphology than in infected PK15. Some cells showed cytoplasmic degeneration characterised by ballooning of the cells (Fig45) and many cells had fused forming polykaryons with up to 10 nuclei. Bacteria could no longer be recovered for the supernatants of PKmi at 9 days.

After 10 days of infection PK15 cells remained in isolated patches and were abnormal with grossly enlarged cytoplasm and nuclei. There was total detachment of cells from the coverslips after 11 days of infection and bacteria were still absent from the supernatant fluids. In infected PKmi monolayers more cells after 10 days remained attached to the coverslips although only a few of these were normal cells. After 11 and 12 days the number of cells remaining attached to the coverslips had decreased and total detachment of

Fig. 44. PKmi cells 1 hour after infection with CSM-C. Extensive bacterial attachment at the periphery of the cell can be seen.

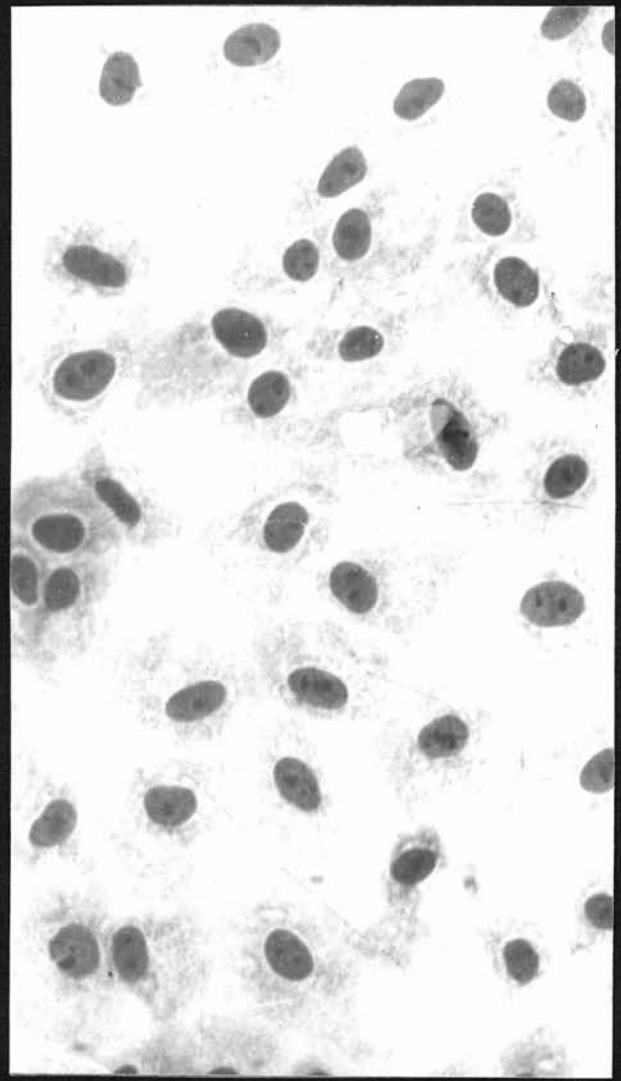
Giemsa stain (x 180)

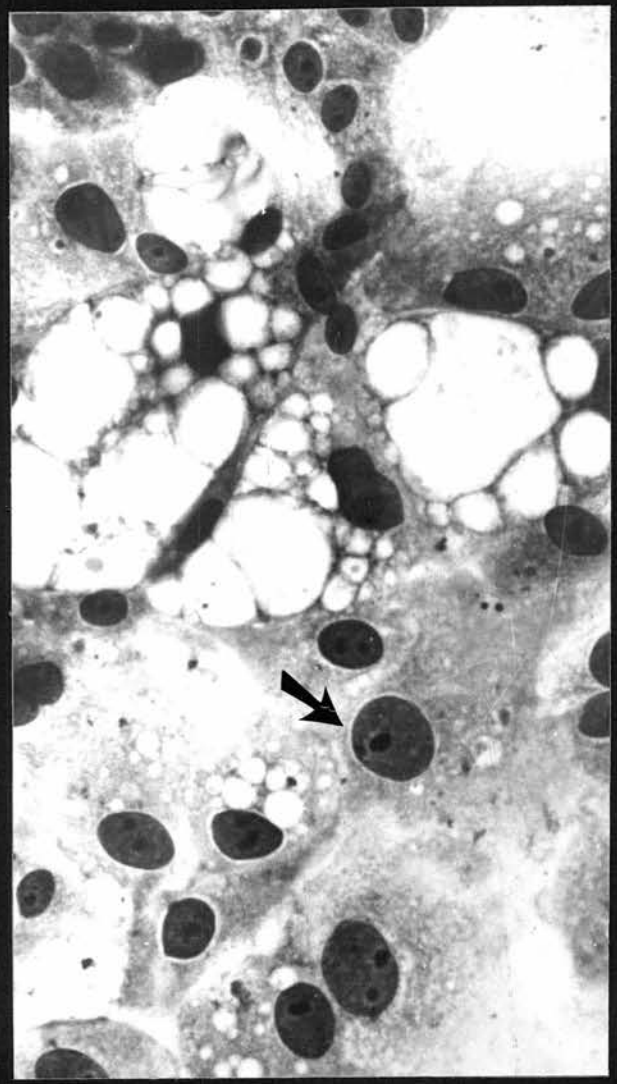
Fig. 45. PKmi cells 7 days after infection with CSM-C. Massive cytoplasmic vacuolation and enlarged nuclei (arrowed) are noticeable.

Giemsa stain (x 113)

Fig. 46. Uninfected PKmi cell monolayer 7 days old.

Giemsa stain (x 113)





cells occurred after 13 days of infection.

8.4.4 Comments

Examination of PKmi cells prior to infection did not reveal intracellular CSM antigen nor were bacteria recovered from the culture fluid. Despite this, however, there were aspects of the cell behaviour following infection with CSM that differed from that of the parent PK15 cells. First, PKmi cells appeared to withstand the effects of exposure to CSM better than PK15 cells since it took longer for the first cytopathic changes to appear in the former. Furthermore, the last patches of abnormal cells in infected PKmi were completely detached from coverslips after 13 days of infection while in PK15 this occurred after 11 days. These changes appear to be due to the increased number of "non-permissive" cells in PKmi monolayer which affect the pattern of attachment and thereafter infection. The marked reduction of CSM in the supernatant fluids of infected PKmi cells after 24 hours, may be due to an increased number of non-permissive cells present in this cell line which reduce the number of cell-associated organisms.

8.5 Discussion

The possible contribution of bacteria in the extracellular fluids of infected PK15 cell cultures to the production of cytopathic changes was shown by the persistence of a cell line (PKmi) after the elimination of the "extracellular" bacteria by vigorous washing of the infected monolayer. A progressive decrease in the number of intracellular organisms took place in passaged cells until no bacteria or antigen were seen after 93 days of

infection and this was associated with the growth of normal cells in place of affected abnormal cells. This continuous decrease in the amount of intracellular antigen with time could be due to the damaging and lethal effect of a brief increase in cell oxygen brought about by vigorous washing of infected monolayers. Thereafter viable bacteria appeared to be largely eliminated and the antigen detected would appear to have been non-replicating organisms.

PK cell lines that have been infected with CSM do not show bacterial attachment when re-exposed to CSM infection (Rajasekhar, 1981). At this stage of post-infection the cells must contain intracellular antigen which may relate to the absence of receptor sites at the cell surface for bacteria of a second infection. PKmi cells although previously exposed to CSM have eliminated viable bacteria and cell antigen; such cells have regained their susceptibility to CSM attachment and the pattern of attachment is similar to that seen in the original PK15 cells. This provides strong evidence that the change in receptor sites is intimately and specifically associated with the presence of intracellular antigen. Despite the absence of CSM antigen in PKmi cells these cells when infected with CSM appeared marginally less susceptible to infection and cytopathic changes than the parent cells. It appears possible that the initial infection resulted in an increase in the number of "non-permissive" cells in the surviving population (PKmi).

CHAPTER 9.

Chapter 9

General Discussion

In this work attempts have been made i) to validate and refine the procedures initially developed by Rajasekhar (1981) for infecting cell culture systems with cultured CSM ii) to investigate the phenomenon of attachment of CSM to cell cultures and cells derived from pig intestine iii) to expose tissue culture cells to infection by campylobacters derived directly from pathologicical specimens iv) to search for the presence of additional campylobacters or other as yet undescribed bacteria in the lesions of PIA that might also infect cultured cells and v) to examine cultured cells infected with inoculum derived directly from affected tissues for the presence of agents other than bacteria. It was hoped that these in vitro studies might disclose features of cellular infection that have remained hidden using conventional bacteriological techniques and so lead to a better understanding of the pathogenesis of the proliferative enteropathies. Rajasekhar (1981) evaluated the growth characteristics of CSM in different types of cell cultures and found that pig kidney cells showed the largest number of attached CSM and smallest number of refractory cells in a population than other species of cell cultures. It was for this reason that most of the experiments in this present work were performed with PK15 cells.

Lawson and co-workers have contributed to our present knowledge of porcine proliferative enteropathies, however some crucial aspects of this disease complex remain to be understood. For example, campylobacter-like organisms have been constantly demonstrated within the cytoplasm of adenomatous epithelium and CSM has been isolated in large numbers from affected tissues, exposure of neonatal or post-weaned conventional pigs to

CSM results in only limited colonisation of the alimentary tract and the disease condition is not produced (Roberts et al, 1980a, b). Exposure of neonatal pigs to CSM and affected mucosa obtained from the natural disease has apparently resulted in transmission of the disease (Roberts et al, 1977). Unfortunately however the experimental conditions in this last investigation were not fully controlled and as repeat experiments with adequate controls were unsuccessful the method cannot be recommended for regular transmission of the disease under experimental conditions. In addition exposure of gnotobiotic piglets to CSM or CSM and rotavirus resulted in both oral and intestinal colonisation by the campylobacter but cultural, histological and ultrastructural studies failed to show the presence of significant intracellular bacterial parasitism or adenomatosis (McCartney, et al, 1984). Although Lomax et al (1982a, b) claim to have reproduced the disease in both specific pathogen-free and caesarian-derived colostrum-deprived pigs with diseased mucosa the reported results are superficial in detail and this work has to be repeated and extended to confirm and further assess the results. In view of these observations and in the absence of any clear cut method by which the condition can be investigated, it seemed that a better understanding of the relationship between the bacteria and cell culture systems might yield information of value in elucidating some of the features that influence the production of the disease in the pig.

Attachment of enteropathogenic bacteria to the mucosal surface of intestinal epithelial cells is an important determinant of virulence for some organisms (Jones and Freter, 1976; Isaacson et al, 1978) such as E. coli, V. cholerae and Shigella spp. In this present work the attachment of CSM to PK15, MDBK and Int 407 cells has been demonstrated. The adhesive properties of CSM appear to be complex and differ from those of some other

Gram-negative bacteria in that this organism does not possess any cell surface appendages such as fimbriae or fibrillar antigens which are known to be involved in bacterial adhesion to cell surfaces. Experimental evidence obtained in this study suggests that active motility of CSM plays an important role in enhancing bacterial attachment to the surfaces of permissive cell cultures, although the polar flagellum did not appear to act as an adhesive appendage. The importance of bacterial motility in the adhesive process of *V. cholerae* has been well documented in both *in vitro* and *in vivo* systems and non-motile variants are invariably non-adhesive (Freter and Jones, 1976; Jones and Freter, 1976; Guentzel and Berry, 1975). Previous workers (Rajasekhar, 1981; Newell and Pearson, 1981) have suggested that bacterial motility is involved in the attachment of some campylobacters to cells in culture.

Preformed monolayers of PK15 cells exposed to CSM showed bacteria adherent to some 95% of the cells during the peak period of attachment. Although, the weight of bacterial inoculum, cell to cell contact and stage in cell growth at the time of infection, have been shown to be partly responsible on occasion for the refractory nature of cells, these factors did not fully explain why bacteria did not adhere to a small number of cells in the "standard" infection model. It may be that these refractory cells are or remain immature and do not develop receptor sites for the attachment of CSM. The infecting dose of CSM has an effect on both the number of organisms that attach to the cells and the number of cells that show bacterial adhesion. Where the bacteria/cell ratio is high permissive cells will be exposed to adequate numbers of organisms and hence almost all cells exposed show large numbers of attached bacteria. Although the use of cells in suspension compared with those grown in monolayer indicated a difference in the number of cells showing bacterial attachment which may

be due to abolition of cell contact, too much emphasis should not perhaps be placed on these differences as suspension culture itself may possibly modify receptor sites. PK15 cells did not show attachment during the first 8 hours of growth but maximum attachment was attained after 20-24 hours of growth. The failure to detect a stage of non-attachment of CSM in synchronised cells does not indicate that the failure to attach bacteria during this period is related to the stage in cell growth at the time of infection since bacteria adhered to synchronised cells during the first 8 hours of growth. However, it is possible that the cell surface is modified by hydroxyurea resulting in alteration or activation of receptor sites which are otherwise inactive in the early stages of growth of untreated cells. The pattern of attachment of CSM to PK15 cells exposed to hydroxyurea was denser than untreated cells and experiments with cells exposed to 1 mM hydroxyurea for 1 hour showed that hydroxyurea exerted this effect on attachment at the level of receptor sites on the cell surface, since this concentration of hydroxyurea did not synchronise cells.

The attachment of CSM to E. coli $K88^+$ adherent and non-adherent pig brush borders was much less intense than the adherence to PK15 cells. The receptor sites for CSM adhesion on pig brush borders or PK15 cells are either different or considerably less numerous in the former because adherence to brush borders is minimal in comparison with PK15 cells. Brush borders prepared from 40 pigs, (both K88 adhesive and non-adhesive) showed similar results and it therefore seems unlikely that the attachment of CSM to intestinal epithelial cells of other pigs will be materially different. This experimental evidence therefore suggests that further transmission experiments with genetically different gnotobiotic pigs would not be justified. Although the demonstration of the ability of CSM to attach to cell cultures including pig kidney cells has indicated the mechanism by which the organism may gain access to the enterocyte, the scanty

attachment of CSM to pig brush borders *in vitro* may be involved in the irregular transmission of the disease in experimental animals. It may however merely indicate that crypt cells may not behave in a similar manner to mature enterocytes in respect of the attachment of bacteria. Logically the infection of crypt cells is likely to be more crucial to the development of mucosal infection than infection of enterocytes as it is in the former area that cell division takes place and from which widespread mucosal parasitism can develop.

Transmission and scanning electron micrographs of pig kidney cell culture inoculated with CSM showed that the bacteria closely adhered to the cell surfaces and there was little separation between the outer bacterial surface and the cell membrane. There is considerable experimental evidence which indicates the importance of bacterial cell surface components in the adhesive process (Smith, 1977; Gibbons, 1980; Ward and Berkeley, 1980). Determinants on both or either host cell and parasite surfaces involved in the attachment of CSM to cell cultures are only poorly understood but the evidence obtained from this work indicates that N-acetyl-D-galactosamine and N acetyl-glucosamine moeities on the cell surfaces play a part in the adhesion of CSM to PK15 cells. Carbohydrate groups have been shown to play critical roles at the interface between other parasitic bacteria and their hosts. For example, L-fucose in the adhesion of *Vibrio cholerae* to brush borders (Jones and Freter, 1976), D-mannose in the adhesion of *Candida albicans* to human buccal cells (Sandin *et al*, 1982) and N-acetyl-D-glucosamine in the adhesion of *Chlamydia psittaci* to mouse fibroblasts (Levy, 1979). Neuraminic acid containing mucopolysaccharide of cell surface receptors do not appear to be associated with CSM attachment to PK15 cells since RDE treatment of cells did not prevent the adhesion of bacteria to cells. α-D-mannopyranosyl and α-D-glucopyranosyl

residues also do not seem to play any part in CSM attachment to PK15 cells. Rajasekhar (1981) reported the reduced attachment of CSM to PK_{pi} cells pretreated with 50μg/ml con-A. Since these cells are pig kidney cells that have an altered cell surface due to persistent infection with Newcastle disease virus this difference in result with this lectin may be related to the alteration of cell surface. Only anti-CSM serum was able to completely block the attachment of CSM to PK15 cells. The antiserum was prepared against live bacterial cells and therefore contains antibodies to most of the bacterial surface components. The specific nature of CSM attachment to PK15 cells is further evidenced by this result. Other workers have reported an inhibitory effect of immune serum on attachment of other bacteria to tissue culture cells. For example, homologous antisera prevented the adhesion of Bordetella pertussis to human fibroblasts (Holt, 1972) and convalescent rabbit immune serum blocked the adhesion of Treponema pallidium to cultured rabbit testicular cells (Fitzgerald, et al 1977). Attempts to definitively identify the chemical nature of the host parasite surface groups involved in the interaction between PK15 cells and CSM did not yield a clear cut result, and the complexity of the process is illustrated by this study. Failure of a specific procedure to alter binding suggests however that the target groups play no role in attachment or that multiple sites exist, however, resistance of substrate to modification or even the generation of new binding sites cannot be ruled out. Likewise reduction of binding following specific treatment suggests that the target group is required for attachment, however, the role of the groups may be only indirectly related to the actual receptor site.

The phase of bacterial attachment is invariably followed by phagocytosis of CSM and electron microscopic evidence suggests that the bacteria do not appear to actively penetrate the cell membrane but are engulfed by the host

cell. The engulfment of CSM appears to be similar to that observed with Neisseria gonorrheae by monkey kidney cells (Waitkins and Flynn, 1973) and Yersinia pseudotuberculosis by HeLa cells (Bovallius and Nilsson, 1975). However in the intracellular location CSM was either present within phagosomes or the bacteria lay free in the cytoplasm unbound by host-cell membranes. Although, experimental evidence has shown that vigorous washing eliminates extracellular bacteria from infected PK15 cells leaving a number of intracellular organisms in the cell monolayers, this method is not successful for all infected cell lines due either to detachment of the monolayer from the glass or persistence of some extracellular bacteria. However, the use of this method to successfully eliminate extracellular bacteria from infected PK15 cells is a better alternative to the use of antibiotics which may introduce problems in the interpretation of results. Viable counts of CSM in infected cell cultures have suggested that intracellular growth of this organism occurs in PK15 and MDBK cells. There is an increase in the number of organisms in infected Int 407 cells which appears to be due to both intracellular and extracellular multiplication of bacteria since during this time of increase bacteria are still attached to the cell surface. In all cell lines the isolation of bacteria from infected cells is associated with the survival of infected cells and as a consequence there is a decrease in bacterial counts in the supernatant fluids and lysates as cells undergo cytopathic changes and the monolayer disintegrates. Other workers have reported intracellular multiplication of organisms in cell cultures determined by surface viable count method of infected cell lysates. For example, Salmonella typhimurium in HeLa cells (Kohlström, 1977), Legionella pneumophilia in monocytes (Horwitz and Silverstein, 1980), in human foetal lung fibroblasts (MRC-5), HeLa, and mouse synovial cells (McCoy) (Daisy, et al, 1981). Evidence for

intracellular multiplication was also provided by immunofluorescence staining of infected cells showing cell-associated bacteria. The progressive increase of intracellular particles in infected cells towards the later stages of experiments was not reflected in the number of viable bacteria suggesting that much of the increase was not in viable bacteria but bacterial antigen and this clearly underlines a major limitation of this method. Several workers have relied on immunofluorescence staining to assay the intracellular growth of pathogenic bacteria (Horwitz and Silverstein, 1980; Daisy et al, 1981). Growth studies demonstrated cell-associated growth but did not indicate that any significant multiplication took place in supernatant fluids of infected cells. The parasitic bacteria present in the supernatant fluids of infected cell cultures are released into the medium after a phase of intracellular growth. This concept is in part based on the fact that refractory cell lines like chicken embryo fibroblasts failed to support parasitic growth (Rajasekhar, 1981) and in this study it was shown in 4.4.5 that PK15 cells failed to support parasitic growth of CSM when infected during the refractory period of cell growth. Furthermore, CSM is rapidly killed in cell-free tissue culture medium.

Bacterial attachment could not be demonstrated on PK15 cells infected with CSM derived from either infected tissue culture cells or adenomatous tissue in contrast to cells infected with cultured bacteria. Tissue or tissue culture derived CSM are less motile than cultured CSM in suspension, this coupled with the low infecting dose from these sources may explain why bacterial attachment could not be visualised. Tissue culture derived CSM did not produce any cytopathic changes in PK15 cells nor could it be serially propagated in the cells. In the present investigation the available experimental evidence suggests that the number

of organisms in the inoculum is crucial to the production of cytopathic changes and the propagation of the bacteria in PK15 cells.

Three bacterial types (1, 2 and 3) were isolated from homogenised PIA tissue and limited observations failed to show the presence of any virus particles. The purified inoculum prepared from PIA homogenate by dilution and filtration either contained apparently only one bacterial type (3) which has been shown to be CSM or that this inoculum contained another organism which could not be cultured on conventional bacteriological media. Catalase positive type 2 which is a campylobacter or type 1 which is not a campylobacter each multiplied in the extracellular fluid of infected cells and caused total destruction of the monolayers within 2 days of infection making it impossible for the changes produced by these isolates to be investigated further. Several workers (Lawson and Rowland, 1974; Rowland and Lawson, 1975; Gebhart et al, 1983) have isolated catalase positive campylobacters C. coli, C. jejuni, C. fetus ss fetus, or C. hyointestinalis from the intestines of pigs with proliferative enteropathy. The production of rapid degenerative changes in tissue culture cells by catalase-positive campylobacters have been reported by other workers. C. hyointestinalis (124/73 A4) produced degenerative cytopathic changes in both primary and established pig kidney cells after 24 hours of infection and by the second day there was total destruction of the cells (Rajasekhar, 1981), HeLa 229 and Int 407 cells exposed to C. jejuni showed the presence of cytopathic changes evidenced by dead and dying cells after 18 hours of infection (Newell and Pearson, 1981).

Light microscopy of Giemsa-stained and unstained preparations of cells infected with inoculum containing type 3 bacteria (CSM-T) or cultured CSM (CSM-C) showed that bacteria from both sources produced similar cytopathic changes in PK15 cells. However, transmission electron micrographs

of CSM-T infected cells showed an increase of intracytoplasmic filaments during the later stages of the infection. These changes in the opinion of several workers is a sign of regressive or degenerative changes resulting from ageing or noxious influences and have been noted in chondrocytes of rabbit articular cartilage associated with ageing (Barnett et al, 1963) and synovial intimal cells in rheumatoid arthritis (Ghadially and Roy, 1967). The increase in the number of organisms in the supernatant fluids of cells infected with CSM-T after 3 days of infection appeared to be a characteristic of tissue derived CSM and related to its infectivity or pathogenicity. There was no evidence that this increase was the result of the presence of another organism in the filtrate that could affect the growth of CSM. Tissue-derived CSM could not be serially passaged in PK15 cells. This suggests that once CSM-T has infected tissue culture cells it loses its virulence and this together with the low number of organisms in the inoculum makes it impossible to serially propagate CSM-T infection in the cells. Identical results have been reported by Jacoby and Johnson (1981) working on CER cells in culture infected with mucosal suspensions of affected hamster ilea. Infected cells were passaged at weekly intervals and they ceased to be infectious for new CER cultures after 2 passages. The inability to reproduce adenomatosis in gnotobiotic piglets using cultured CSM might be due to alteration in the pathogenicity of CSM-C through repeated subculture. It was possible to initiate successful tissue culture infection with an inoculum of as low as 3.4.8 $\log_{10}$ organisms per ml of CSM-T which was not possible with an infecting dose of 5.26 $\log_{10}$ organisms per ml of CSM-C. Filtrates containing only campylobacter-like forms but no viable bacteria, derived from PHE proved non-infective for PK15 cells. The inoculum from PHE tissue capable of passing through larger pore diameter filters contained catalase-positive organisms

with the characteristics of Campylobacter coli/jejuni groups. These bacteria multiplied in the extracellular fluids of infected tissue culture cells and caused the destruction of PK15 cells with 2 days of infection. Experimental evidence has demonstrated that the intracellular organism present in PHE tissues is CSM and the most convincing explanation is that the bacteria have been possibly altered and cannot be recovered on conventional media (Lawson et al, 1979). However, CSM has been recovered on occasion from the lesions of PHE in numbers sometimes similar to those observed in tissues (Lawson et al, 1979; Love and Love, 1979).

It has been shown by Lawson et al (in press) that serum from rabbits immunised with bacteria extracted from the lesions of PHE reacts with intracellular Ω campylobacter-like antigen present in the cells of proliferative enteropathies of the pig and the hamster. It appears that a similar Ω antigen is present in PK15 cells infected with CSM-C or CSM-T because these cells fluoresce with RIΩS while this antigen is not detected in uninfected control PK15 cells. Similar results have been reported by Jacoby and Johnson (1981) in primary hamster embryo cells infected for 1 week with suspensions of affected hamster ilea. These cells contained intracytoplasmic rod-shaped bacteria that reacted with anti-TIH serum (serum from affected or exposed hamsters) but not with normal serum or hyperimmune serum to conventional intestinal organisms.

A once-infected PK15 (PKmi) cell line was established which contained no residual bacteria or antigen after 93 days of infection (14 passages). The attachment of CSM to PKmi cells suggests that the receptor sites for adhesion of the bacteria had been replaced following CSM infection of cells. PKmi cells infected with CSM resisted destruction and detatchment longer than infected PK15 cells. This appears to be a consequence of the PKmi cells population containing an increased number of

refractory cells which may influence the production of cytopathic changes following infection of cells.

In conclusion, the experimental evidence obtained in this study suggests that tissue culture derived bacteria are not likely to be more effective than cultured CSM in the production of the disease in experimental animal infections. Loss of virulence or poor adaptation to intracellular growth are possible reasons for the failure to induce disease with cultured bacteria. The same is the case with tissue culture derived bacteria used for infection of fresh tissue culture cells. Jacoby and Johnson (1981) reported failure in attempts to transmit transmissible ileal hyperplasia in weanling hamsters with organisms derived from CER cells. CSM derived directly from adenomatous tissue was capable of initiating infection in cell cultures at low concentrations while cultured CSM could not establish infection at this level. This result has shown that CSM-T is more successful than CSM-C in initiating infection and it is surprising that animal experiments with diseased mucosa failed to reproduce the disease regularly although these reproduction attempts have been performed in conventional pigs. Evidence has shown that the attachment of CSM to pig brush borders was scanty compared to PK15 cells and this may affect the reproduction of disease in these experimental animals with established immune systems. Infection experiments should now be performed with filtrates of diseased mucosa in gnotobiotic pigs since these class of animals lack passive immunity. Previous attempts to expose young colostrum-deprived pigs to mucosal homogenates resulted in rapid death (McCartney, 1983) which obscured any possible experimental results. The development of filtration techniques and the demonstration of enhanced pathogenicity for tissue culture of CSM-derived directly from diseased mucosa now makes this experiment a realistic possibility without an unacceptable mortality in the exposed animals.

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